BORNA DISEASE VIRUS, UK PERSPECTIVE

A dissertation submitted

By

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This thesis is submitted by the undersigned to the faculty of Applied Sciences of University of Northumbria in partial fulfilment of the examination in partial fulfilment of the requirement for the degree of Doctor of Philosophy. The author confirms that the manuscript, the title is original and has not been submitted for an award from this or any other institution, either in the United Kingdom or elsewhere.

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Abstract

Borna Disease Virus (BDV) is a single stranded, negative sense, RNA virus and the pathogenic agent of Borna Disease (BD). BD was first described as a disease of horses and sheep, however, it has now has been recognised to cause a persistent infection that leads to neurological and behavioural disturbances in a wide range of hosts, including humans. BDV has been reported in most countries, including most of central Europe, North America, East Africa, East Asia and Australia. However, reports of the incidence and prevalence in both animals and humans in the UK are limited.

This study investigated the prevalence of BDV in both human and horse populations in the UK using a range of serological and molecular methods. 528 human samples (95 patients with mood disorders, 32 healthy individuals in close contact with psychiatric patients and 401 blood donors) and 274 horse samples were screened. The results show that BDV is present in both horses with a seroprevalence of 13 %. For humans in the UK a significantly higher proportion of mood disorder patients (29 %) and healthy individuals in close contact with psychiatric patients (28 %) were seropositive for BDV as compared to normal blood donors (17 %), $\chi^2 = 8.418$ p= 0.015, in this study.

From a public point of view, this data is important as it suggests that BDV may be transmitted from psychiatric patients to healthy individuals who are in close contact with them, although it must be stressed that this study only investigated a small number of individuals. Thus a detailed sero-epidemiological study of BDV prevalence in the UK is needed to determine the distribution of this important human virus.

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Dedication

I dedicate this dissertation to my family, past, present and future, and especially to my wonderful children, Victor, Henrique and Gisel, and husband Gil. It's been a challenging and demanding effort so far. May God compensate us for all the sacrifice we had to go through and make the next several hundred years better than the last few have been.

Thank you.

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A special thanks to Prof. Derek Gardiner, Dr David Holmes, Gary Black, Susan Irving and Linda Barlow for trusting me and for giving me all the support when I needed most. Thank you for pulling me up when I was down, for your enthusiasm, persistence, support and for giving me a cushion for my tears.

I wish to acknowledge my parents. My father (may his soul rest in peace) taught me how to write, for which I will always be grateful. My-step-father, for encouraging and giving me the support to continue my studying, and my mum who taught me to persevere, be a fighter and to smile whether circumstances warranted it or not. I wish to acknowledge my sisters who have been my pillars at all times making sure that in my life journey I never give up. Thank you, without you I would never make it, you are my truly and only friends.

Finally, I acknowledge my God, upon whom I depend upon for my life, and for all that I have and am.

List of frequently used abbreviations:

Ab	Antibody
Ag	Antigen
AP	Alkaline Phosphatase
Ara-C	1-ß-D-arabinofuranosylcytosine
BD	Borna Disease
BDV	Borna Disease Virus
BP	Bipolar disorder
C6	Rat glioma
CIC	Circulating immune complexes
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle's minimum essential medium
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic & Statistical Manual of the Psychiatric Association –revised fourth edition
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FFU	Focus forming unit
GTP	Guanosine-5'-triphosphate
IFA	Indirect immunofluorescence
IgG	Antibody (glycoprotein) with gamma (γ) chains
IgM	Antibody (glycoprotein) with mu (μ) chains
MDCK	Madin-Darby canine kidney
MDD	Major depressive disorder
Moabs	Monoclonal antibodies
NCS	Newborn calf serum
OL	Human oligodendroglial cells
P24	BDV protein 24 (phosphoprotein)
P40	BDV protein 40 (nucleoprotein)
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RC	Rapid cycling
RIA	Radioimmunoassay
RNA	Ribonucleic acid
SDS PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
TAE buffer	Triethanolamine hydrochloride
TBS	Tris buffered saline
$TCID_{50}$	Tissue culture infecting dose
TE buffer	Acronym for Tris buffer (E stands for EDTA)
TEMED	N,N,N,N -Tetramethyl-Ethylenediamine
Tris	Tris[hydroxymethyl]aminomethane
TRITC	Tetramethyl rhodamine isothiocyanate
WB	Western blotting
YRB	Young rabbit brain (Primary cells)
YRS	Young rabbit spleen (Cell line)

CHAPTER 1:

1. INTRODUCTION

1.1. Brief history of BDV

The first report of Borna Disease was in 1767 in the Kingdom of Bavaria and Wűrttemberg by Johann Baptist Von Sind who reported curing horses with "head disease"(Dürrwald & Ludwig, 1997). In the following hundred years or so a number of other reports appeared in medical books of that time narrating the death of horses with similar signs of encephalopathy. A large number of terms describing the disease were used the years and these varied from "sad horse disease", "brain fever", "sub-acute meningitis", "hypersomnia of horses" to "polioencephalomyelitis non purulenta infectiosa" all of which reflect the restriction of the disease to the central nervous system (CNS) (Stitz & Rott, 1999). It was believed that the disease was heritable and occurred mainly in spring, with variable severity in different years and this observation is still made (Staeheli *et al.*, 2000).

In the late 1800's an epidemic outbreak spread in Saxony and for the first time the disease was described as cerebro-spinal-meningitis. The disease led to the death of a very high percentage of horses belonging to the cavalry regiment in the town of Borna in Saxony near Leipzig (figure 1.1) and the name Borna disease (BD) was subsequently adopted. However, Borna was not the first area to be affected by this cerebro-spinal-meningitis and so there was a degree of controversy to this name and it was only finally accepted in the 1970's. Forty years after the outbreak in Borna Schmidt, a German Scientist provided a detailed report on the epidemiology of BD (Richt & Rott, 2001).

In subsequent years further reports appeared in the papers of veterinary services in Germany, resulting from research carried out as a result of the increasing manifestation of disease in central Europe culminating with the first report on sheep infected with BDV in 1896 (Dürrwald & Ludwig, 1997).

In 1926 Wilhelm Zwick and co-workers suggested that the disease was caused by a virus and they were successful in transmitting the disease from infected horses into rabbits and guinea pigs and then back to horses (Richt *et al.,* 1997a). The same brain material from these rabbit passages are still in use today and are named BDV strain V.

In 1978 Hanns Ludwig took strain V to Berlin to use as a reference strain in his laboratory and later adapted this strain to rats and tissue culture. The Borna virions from these cultures served as starting material for the description of the first Borna virus sequence (Briese *et al.,* 1994; Dürrwald & Ludwig, 1997).

In 1964, Von Sprockhoff discovered the complement-fixing antigen involving the soluble antigen (s-antigen) providing the breakthrough for the sero-diagnosis of BD.

In 1985 a study by Amsterdam *et al,* provided the first evidence of BDV in humans, and a possible connection between this virus and human psychiatric disorders, when they observed that a higher percentage of psychiatric patients had BDV specific IgG

as compared to healthy controls. This study and many similar others have prompted a dramatic increase in BDV research and the search for the distribution of this virus (Rott *et al.*, 1985).

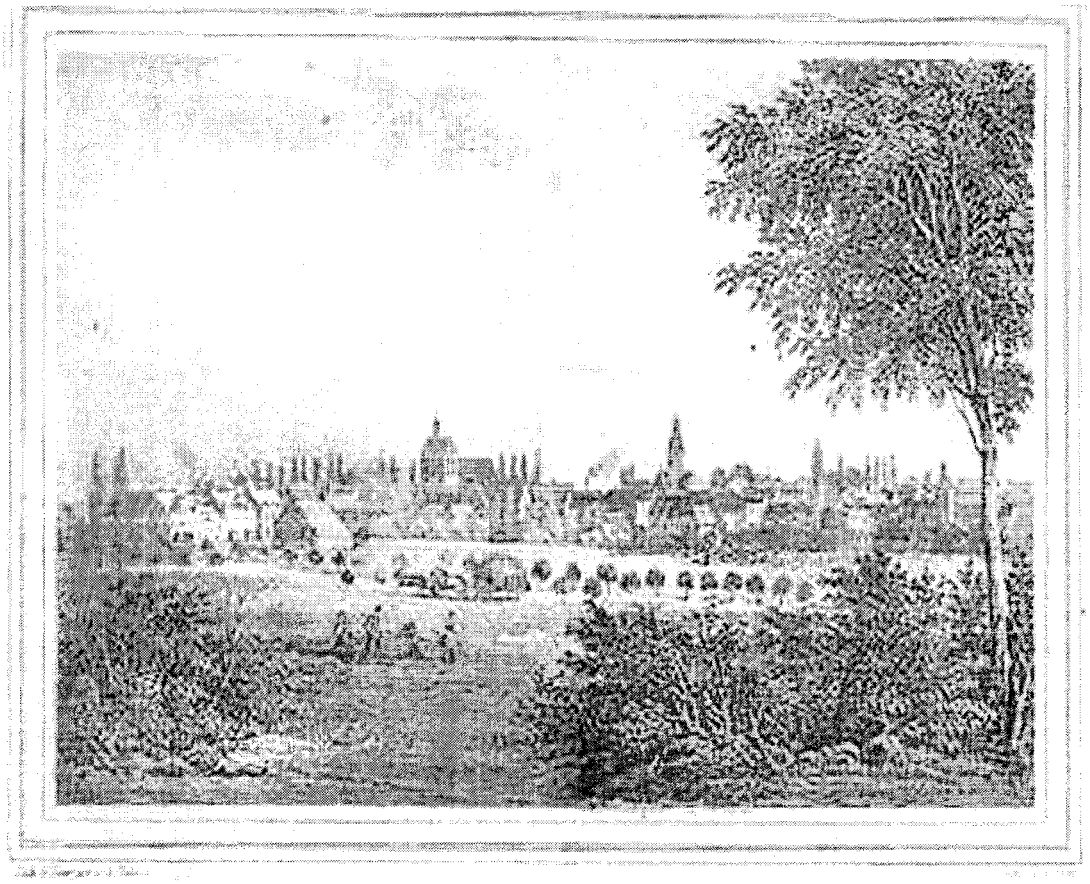

Figure 1.1: Borna. Germany's city where the first scientific evidence on Borna Disease outbreak was recorded. Extracted from Durrwald & Ludwig (1997).

1.1. Borna Disease Virus

Borna disease virus (BDV) is the causative agent of borna disease in a wide range of animals. However, recent reports suggest that BDV plays a critical role in the etiopathology of a number of human neurological and psychiatric disorders including bipolar disorders (Amsterdam *et al.,* 1985; Bode, 1995; Bode *et al.,* 1995).
Borna disease (BD) is a disseminated non-purulent meningoencephalomyelitis with infiltration of mononuclear cells (Richt *et al.,* 1997a; Dürrwald & Ludwig, 1997; Rott & Becht, 1995). BDV preferentially targets the neurons of the limbic system (within the CNS), which are involved in behaviour, memory, and emotions.

In infected individuals, BDV can exist commensally for an undetermined period of time. Subsequently, an unknown process can lead to activation of the virus and then lead to disturbance of information processing, behaviour and mood alterations (Bode *et al.,* 1992).

1.1.1. Classification and structure of BDV

Borna disease virus (BDV) is a non-segmented negative sense single-stranded RNA genome of ~ 8.9 kilo-bases (kb). It is the prototypic and only member of a new viral family named *Bornaviridae*. The borna virus was placed within the order

Mononegavirales along with *Paramyxoviridae (*e.g. mumps, measles and Newcastle disease virus), *Rhabdoviridae* (rabies and infection haematopoietic necrosis virus), *Filoviridae* (e.g. Marburg and Ebola viruses); *Orthomyxoviridae* (e.g. influenza A, B, C virus); *Bunyaviridae* (e.g. bunyamwera virus) and *Arenaviridae* (e.g. lymphocytic choriomeningitis and hepatitis delta virus) (Richt *et al.,* 1993; Ludwig, 1988; de la Torre, 1994; Fauquet *et al.,* 2005).

The BDV infectious particle has a spherical structure that measures 100-130 nm in diameter and contains an outer membrane envelope with two kinds of glycoprotein spikes (GP43 and GP84) (Richt *et al.,* 1993; Zimmermann *et al.,* 1994; Kohno *et al.,* 1999), which are the first determinant of viral tropism as they attach to a cell surface receptor (Gonzalez-Dunia *et al.,* 1998). BDV has a genome that replicates and transcribes in the nucleus of the host (Richt *et al.,* 1992) and several proteins are used for virus expression.

BDV infectivity can be lost very rapidly with temperatures above 56 °C, acidic conditions, below 5, or alkaline conditions, above 12, and infectivity is also lost with treatments involving organic solvents, detergents, formaldehyde and exposure to ultra violet radiation (de la Torre, 2002).

BDV has the unique feature of replicating and transcribing entirely in the nucleus of infected cells and uses the host nuclear splicing machinery to generate some viral

mRNAs (Cubitt & de la Torre, 1994). Thus, the nucleolus is believed to be the site of transcription and replication of BDV RNA complexes (Pyper *et al.,* 1998).

The viral transcriptional mechanisms are complex. The viral genome contains three transcription initiation sites, four transcription termination sites, and three introns. BDV polymerase recognises transcription start signals within the genome, which contains an exclusive motif and may not utilise a primer for RNA initiation (Richt *et al.,* 1992). Molecular studies have shown distinctive features such as the overlap of reading frames, the post-translational processing of RNA splicing, the nuclear phase's indispensable for virus replication and the unusual level of sequence conservation (de la Torre, 1994). Overlap and splicing allows synthesis of proteins not predicted by the primary sequence of the genome by post transcriptional modification of sub-genomic RNAs (figure 1.2). All of this allows an increased versatility of its primary transcripts and possibility for controlled synthesis of new BDV peptides. The nucleotide sequence is found in database accession number L27077 (BLAST).

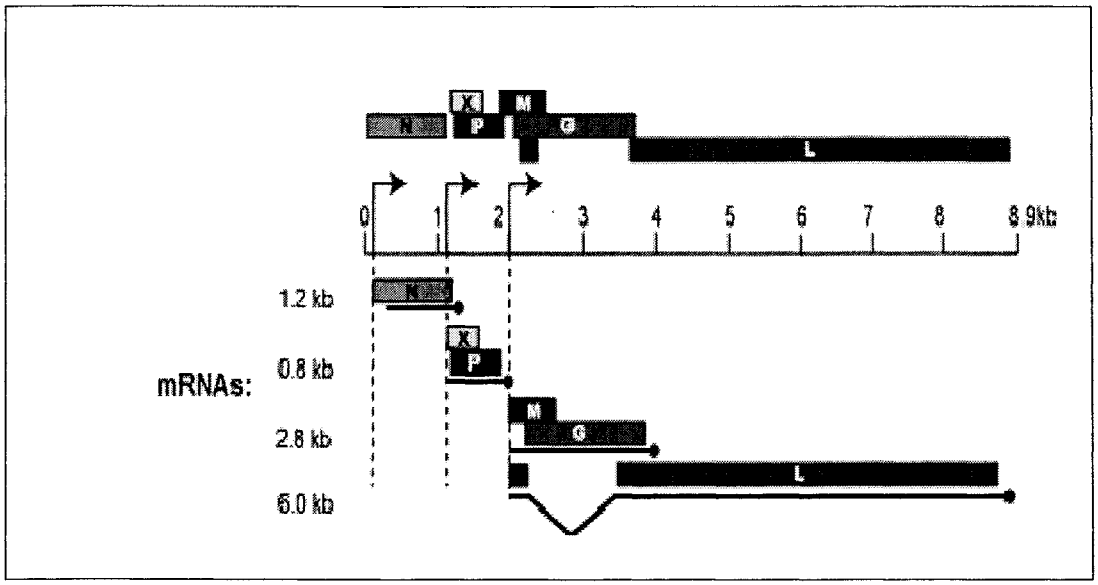

Figure 1. 2: Morphology of BDV genome (taken from Nakamura *et al.,* 1995). Key: N= nucleoprotein (p40); **P** = phosphoprotein (p24); **M** = matrix protein (gp18); **G** = envelope protein (gp94); **L** = RNA-dependent RNA polymerase (p190); **X** = unknown function (p10).

This small size genome (8.9 kb) permits the use of diverse strategies for gene expression and RNA splicing plays an important role in the regulation of BDV genome expression by increasing the versatility of its primary transcripts and by providing the possibility for controlled synthesis of new BDV polypeptides (Cubitt *et al.,* 2000).

The expression levels of BDV proteins have been reported to be higher the closer they are to the 5' end of the genome. Thus nucleoprotein (ORFI) can be found at higher levels than glycoprotein 84 (ORF IV) (Bajramovic *et al.,* 2003). Within the BDV genome six major viral proteins have been identified and partially characterised as open reading frames (ORF) (de la Torre *et al.,* 1996a; Briese *et al.,* 1994;

Gonzalez-Dunia *et al.,* 1998) and they form the basis for the regulation of gene expression and infectivity. They are as follows:

1. The nucleoprotein is about 357/370 amino acids (aa) long and is also designated as p40, N protein or p40/p38 kDa.

This nucleoprotein is encoded by ORF I and is located at the 5′ end of the cRNA corresponding to the BDV-nucleoprotein or nucleocapsid N (38-kDa and 39-kDa). It has a nuclear localisation signal, and is involved in export activities (Tahir *et al.,* 2000). Infected cells contain high levels of this protein, totalling at least 50 % of the viral proteins found, due to its proximity to the 5' end (Pyper *et al.,* 1998).

2. The phosphoprotein or p24 (24-kDa), encoded by ORF II.

The p24 phosphoprotein (201 aa) of BDV is the most prevalent protein found in infected cultured cells and in infected animal brains (Kamitani *et al.,* 2001). This protein corresponds to the BDV phosphoprotein transcription activator P (p24). Although the precise role of the protein in the viral replication cycle has not yet been determined, it has been suggested that P protein is an essential cofactor for viral polymerase (Kamitani *et al.,* 2001; Pyper *et al.,* 1998). With nuclear localisation activity, the P protein is phosphorylated by cellular kinases (Shoya *et al.,* 1998) and may act as a nuclear export factor for BDV (Wehner *et al.,* 1997; Wolff *et al.,* 2000).

3. Matrix protein, also designated as M protein, gp18 or p16 encoded by ORF III.

The M protein (142 aa) is associated with the inner layer of the plasma membrane and, similarly to other homologous proteins (Schneemann *et al.,* 1995; Portela & Digard, 2001; Kraus *et al.,* 2001), this glycoprotein may play a role in neutralising activity (Hatalski *et al.,* 1997; Stayloff *et al.,* 1997), in virion assembly, budding, and in membrane spanning and may act as a bridge between the membrane or G protein and the nucleocapsid. (Stayloff *et al.,* 1998). However, this protein does its job with higher complexity than similar proteins in other non segmented viruses (Kliche *et al.,* 1994; Stoyloff *et al.,* 1997).

4. The envelope glycoprotein, gp84 or gp94, also designated as p56, 57-kDa or G gene encoded by ORF IV.

ORF IV is 503 aa long and results in a BDV-glycoprotein (GP). BDV glycoprotein is expressed as two products of 84 and 43kDa and designated as gp84 and gp43. The first product corresponds to the full length of BDV glycoprotein, while the latter correspond to the terminus of BDV GP. They have two distinct functions. Gp84 is involved with attachment to the cell surface receptor and gp43 is involved in pH-

dependent fusion after internalisations of the virion by endocytosis (Gonzalez-Dunia *et al.*, 1998). The ORF IV overlaps with matrix protein in a different frame (Zimmerman *et al.*, 1994) and may be involved in viral entry (Gonzalez-Dunia *et al.*, 1998; Schneider *et al.*, 1997), in the fusion of the viral and cellular membranes after internalisation of the virion by endocytosis (Rich *et al.*, 1997a; Gonzalez-Dunia *et al.*, 1998) as well as establishment of persistence (Gonzalez-Dunia *et al.*, 1998). Although its expression is limited and poorly recognised by serum antibodies (Gosztonyi & Ludwig, 1995), G protein is also essential for the neutralisation of the virion (Stoyloff *et al.*, 1998).

5. The RNA-dependent RNA polymerase or L protein, also known as p190 or 180-kDa protein encoded by ORV V.

ORF V is an RNA-dependent RNA-polymerase (L polymerase) of BDV located at the 3′ end of the cRNA. It is more than 1700 aa long (de la Torre *et al.*, 1994; Schneemann *et al.*, 1995; Walker *et al.*, 2000). The L protein has a well-conserved putative catalytic domain (Briese *et al.*, 1994; Cubitt *et al*, 2001) and is expressed from the third transcription unit. It is dependent on a splicing event that fuses a small upstream ORF that overlaps with the 5' end of the G ORF (Walker *et al.*, 2000). The protein is present in the nucleus together with the phosphoprotein (Walker *et al.*, 2000). BDV L protein was also shown to be phosphorylated by cellular kinases and interacts with the P protein (Walker *et al.*, 2000).

6. The X protein or p10, encoded by ORF VI.

The last ORF has 87 aa and overlaps with p24 but is coded in a different frame (Wehner *et al.,* 1997). It is located in the nucleus of infected cells but shows cytoplasmic localization in the absence of other viral proteins. The X protein binds directly or indirectly to P and N (Horning *et al.,* 2003; Shoya *et al.,* 1998). The function of X protein is unclear, but it has been suggested to promote nuclear targeting of X protein in infected cells, and might play a role as a cofactor for the viral polymerase (Naegele *et al.,* 2003; Wolff *et al.,* 2000).

1.1.2. Natural and experimental BDV infection

1.1.2.1. Geographical distribution of BDV and host range

Extensive epidemiological studies in both animals and humans have shown that BDV occurs in a number of countries/continents including Japan (Bahmani *et al.,* 1996; Kao *et al.,* 1993; Nakamura *et al.,* 1995), Iran (Bahmani *et al.,* 1996), Israel (Malkinson *et al.,* 1995), Europe (Ludwig *et al.,* 1973; Hagiwara *et al,* 1996), Africa and USA (de la Torre, 1994). Thus BDV is prevalent across a wide geographical area

and it is probably found in many other countries (Kao *et al.,* 1993; Herzog *et al.,* 1994; Nakamura *et al.,* 1995; Bahmani *et al.,* 1996; Hagiwara *et al.,* 1996 and Hagiwara *et al.,* 1997).

Increased reports of BDV in this broad geographical area and the increase in host range might be due to the spread of the virus or possibly due to an improved understanding of the agent and improved diagnostic methods.

Seasonal distribution of Borna disease has been described in horses in central Germany where there is a significantly higher incidence in April, May and June (Staeheli *et al.,* 2000). BD also seems to be more frequent in some years than in others; therefore, arthropods have been discussed as a potential vector (Degiorgis *et al.,* 2000). BDV, however, has never been isolated from insects in Europe although in the Near East, ticks have been associated with transmission of an equine encephalomyelitis similar to BD (Daubney & Mahlau, 1967).

In addition BDV infects naturally and experimentally a diverse range of animal species ranging from birds to primates. BDV antigen, antibodies and/or viral nucleic acids have been detected in the CNS of donkeys, deer, alpacas, mules, llamas, cattle, gerbils, goats, rabbits, dogs, cats, foxes, lynx and ostriches with neurological diseases (some with strong lymphocytic infiltrations of the CNS) (Berg & Berg, 1998; Caplazi *et al.,* 1999; Zimmermann *et al.,* 1994; Hausmann *et al.,* 2005; Staeheli *et al.,* 2000; Caplazi *et al.,* 1999; Caplazi *et al.,* 1994; Bode *et al.,* 1994; Berg *et al.,*

2001; Malkinson *et al.*, 1993; Dauphin *et al.*, 2001). These markers have also been found in cats with staggering disease in the UK (Reeves *et al.*, 1998). However, there is accumulating evidence that the natural host range is wider than implied based on the detection of the viral protein in a range of asymptomatic animals (Degiorgis *et al.*, 2000).

Furthermore the fact that BDV markers have been found in human brains (de la Torre *et al.*, 1996) the similarities between BDV induced behavioural abnormalities in animal models and a range of human mental disorders suggests that BDV might also infect humans (Carbone, 2001). The suggestion that human disease is caused by BDV has been supported by extensive epidemiological studies using both serological and molecular based approaches (Bode *et al.*, 1988). BDV has been found at higher frequencies in neuropsychiatric patients (66.7 %) and in patients with chronic fatigue syndrome (24 %; Nakaya *et al*, 1996) as compared with healthy controls (0-11 %; Rott *et al.*, 1985; Igata-Yi *et al.*, 1996). In support of this supposition a number of other virus such as influenza, herpes simplex virus, retroviruses, and cytomegalovirus (Lewis *et al.*, 1999) have been already implicated in a number of mental disorders. Further clinically diagnosed neurologic disturbance and BDV reactive antibodies have been reported in specific occupational groups such as farm workers (Hatalski *et al.*, 1997; Durrald & Ludwig, 1997; Richt *et al.*, 1992; Weisman *et al.*, 1994; Bechter *et al.*, 1992; Rott *et al.*, 1991; Nakamura *et al.*, 1997) suggesting that BDV may be a potential new zoonotic agent.

1.1.2.2. Transmission of BDV infection

It has been suggested that BDV is transmitted orally, via the trigeminal nerve due to direct contact with secretions or by exposure to contaminated food or water (Sierra-Honigmann *et al.,* 1993) because BDV-specific RNA has been found in saliva, nasal secretions and conjunctival secretions (Becht & Richt, 1996; Schneider *et al.,* 1997; Richt *at al.,* 1993). It has also been proposed that there may be an olfactory route for transmission as intranasal infection seems to be efficient in animals as nerve endings in the nasal mucosa are readily accessible to the virus. This is supported by the observation that naturally infected horse olfactory bulbs are inflamed and oedematous early in the course of disease (Ludwig *et al.,* 1988) and similar observations have been seen in experimental animals after intranasal infection where the infecting virus migrated intraaxonally from the neuroreceptors in the olfactory epithelium into the brain (Narayan *et al.,* 1983b; Sauder & Staeheli, 2003). On Immunohistochemical (IHC) examination these animals showed specific regions of the brain infected by the virus including the ventral hippocampal commissure and the hippocampal CA3 region, the olfactory tubercle, anterior olfactory nucleus, olfactory bulb, septum, fornix, and thalamus (Sauder & Staeheli, 2003), suggesting an important route of transmission. Some of the infected areas are shown in figure 1.3.

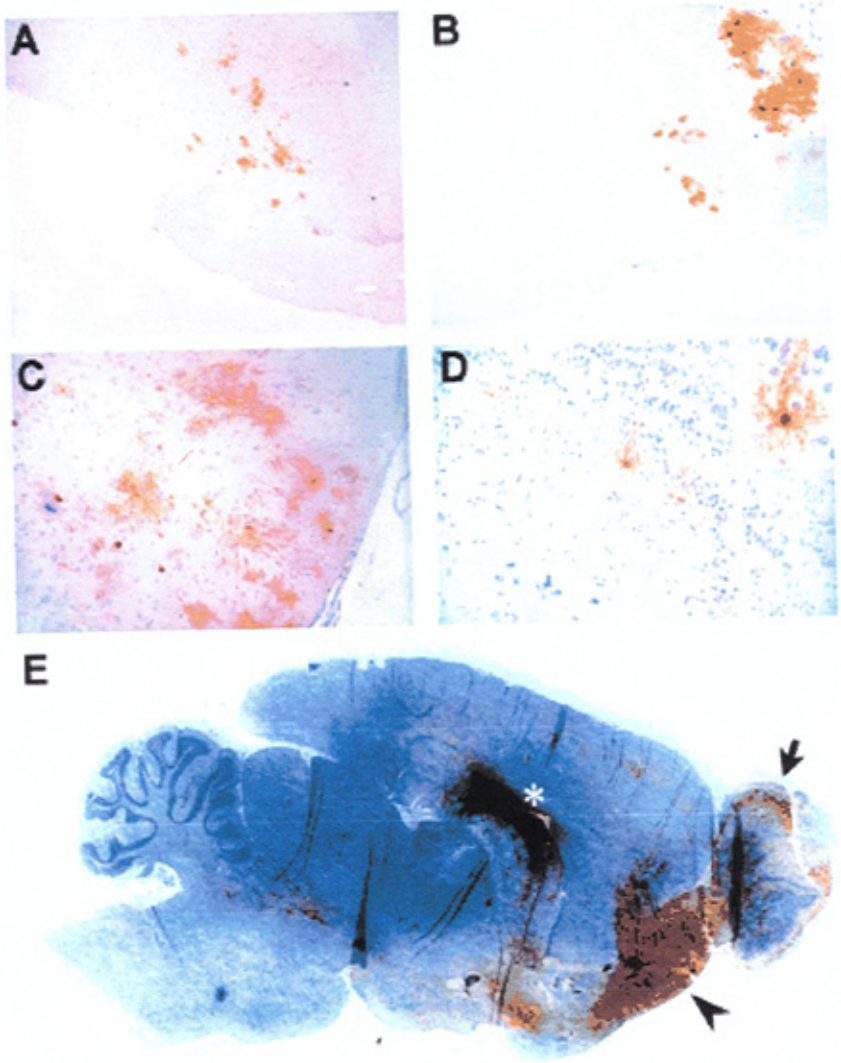

Figure 1.3. Identification of the route of BDV spread in brains of rats. Sections of paraformaldehyde-fixed, paraffin-embedded brains were subjected to IHC to detect BDV-infected cells with BDV-N-specific monoclonal antibody Bo18 (brown stain). Sections were counterstained with Mayer's hematoxylin. (A and B) Two immediately adjacent brain regions of one section. BDV-positive cells in panel A are located in the area of the olfactory tubercle and the ventral pallidum. BDV-positive cells in panels B and C are located in the anterior olfactory nucleus. (D) Part of the olfactory bulb. (E) Ventral hippocampal commissure (asterisk), the region of the anterior olfactory nucleus (arrowhead), and the olfactory bulb (arrow). Brain regions were identified based on maps of Paxinos and Watson. (B and D insets) BDV-positive neurons at a higher magnification. Extracted from Sauder & Staeheli, 2003.

BDV nucleic acid and proteins can be detected in peripheral blood mononuclear cells and this indicates the potential for haematogenous transmission although at the moment there is no evidence for this (Sierra-Honigmann *et al.*, 1993; Rubin *et al.*, 1995). After the virus has entered the nervous system it migrates to the CNS, where it replicates in neurons and astrocytes and with time the virus spreads throughout the CNS (Stitz & Rott, 1999).

It has also been suggested that rodents may serve as a reservoir as well as vectors for virus dissemination and it has been observed that they can be persistently infected with BDV and excrete the virus in urine (Hagiwara *et al.*, 1997). There is also an increased prevalence in the hotter seasons and in facilities with poor hygiene (Tsujimura *et al.*, 1999; Staeheli *et al.*, 2000). However the precise route of natural transmission of BDV has not as yet been elucidated and the role of potential animal reservoir of disease is still to be fully investigated. In addition there is no data available as to potential vertical transmission of BDV in natural or experimental infections.

1.1.2.3. BDV infectious cycle and replication

It is believed that BDV enters cells through the endocytotic pathway and is mediated by a decrease in pH which allows the initiation of infection (Figure 1.4). This acidic environment is achieved by the presence of gp43. This expressed protein (gp43) contains the hydrophobic N terminus that provides the pH dependent fusion for a BDV productive infection (Gonzalez-Dunia *et al.,* 1997). Figure 1.4 shows a diagram of BDV infection and replication.

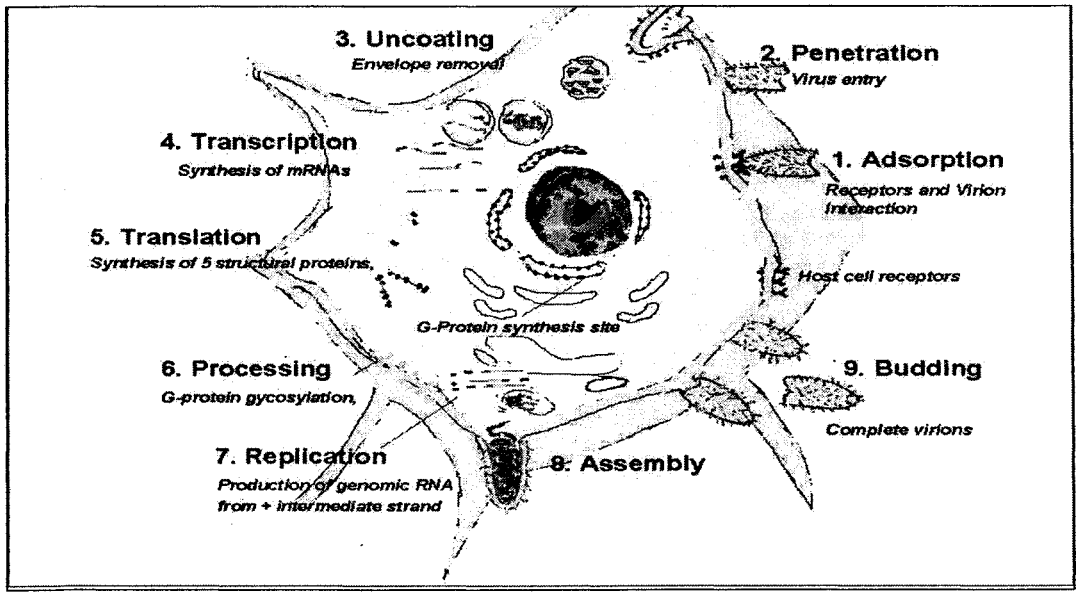

Figure 1.4: Cell cycle of infection and replication. A representative diagram of viral binding and penetration into cell, where in the binding stage phospholipids act as the cell surface receptor molecule and at the penetration stage the nucleocapsid enters the cytoplasm. All subsequent stages take place here with no involvement of the nucleus of the cell (modified from Conzelman, 1998). Note that although the replication and translation of BDV occurs in the nucleus, this diagram shows it occurring in the cytoplasm for clarity purposes.

In general all members of *mononegavirales* have different strategies for entering cells. Some members have two surface receptors (i.e measles virus) with independent distinct functions. There are others (*Paramyxoviridae*) with two receptors that pH-dependently need to first fuse to form one receptor and subsequently fuses to the plasma membrane (Paterson *et al.*, 1984; Lin & Lamb, 2000). However, some members have only one surface glycoprotein (i.e. rabies virus), which on its own is capable of accomplishing attachment, fusion upon endocytosis and endosomal acidification (Mebatsion *et al.*, 1996). Similar to rabies virus, BDV express a single protein on its surface (gp84) and it has been suggested that viral penetration and fusion involves this viral gp protein as well as the M proteins (Gonzalez Dunia *et al.*, 1997). The L protein (ORF V) is the first to have effect when entering the cells. It processes individual mRNAS for each one of the six open reading frames. Thus, by supplying the polymerase enzyme, RNA is made and viral protein synthesis proceeds. The sequence of transcription goes from N to L (see figure 1.2) and thus due to its transcription place (last) less L mRNA is made than any other protein, thus the earlier they are made the higher the amount of the viral protein production.

After the polymerase has transcribed the negative-sense genomic RNA into a positive sense strand it is used as a template for the transcriptase to transcribe new negative sense genomic RNA molecules and requires protein synthesis with the same polymerase and is controlled by the N protein.

At assembly (stage 8) the G protein mRNA is translated in association with the endoplasmic reticulum and transported via the Golgi body to the cell surface.

Once it reaches the cell surface BDV requires cell to cell contact for its spread despite a considerable low virus release at assembly (Carbone *et al.,* 1993). The suggestion is that complete virions then bud and disseminate through the neuronal synaptic connections and from that to other areas of the central nervous system (Morales *et al.,* 1988). This is supported by observations that BDV antigen distribution in CNS via cell to cell dissemination may be possible through synaptic contacts between neurons (figure 1.5). The mechanism by which BDV disseminates via these synaptic contacts is not clear.

It has been shown that after experimental nasal inoculation of BDV into a rat the virus replicates in the olfactory epithelium and then migrates to the main olfactory bulb which is the first central relay for olfactory input, where the first-order sensory neurons contact the second-order sensory neurons, the mitral and tufted cells (Greer & Shepherd, 1982). The neurons from the lateral olfactory tract project to structures of the limbic system (many concerned with motivation, emotion and certain kinds of memory) the amygdala, septal nuclei, pre-pyriform cortex, the entorhinal cortex, hippocampus and the subiculum. It also projects to the "pleasure centres" known as the septal nuclei and amygdale. The hippocampus is concerned with motivational memory (the association of certain stimuli with food). Projections are also sent to the thalamus and from there to the frontal cortex for recognition and all are susceptible to BDV infection.

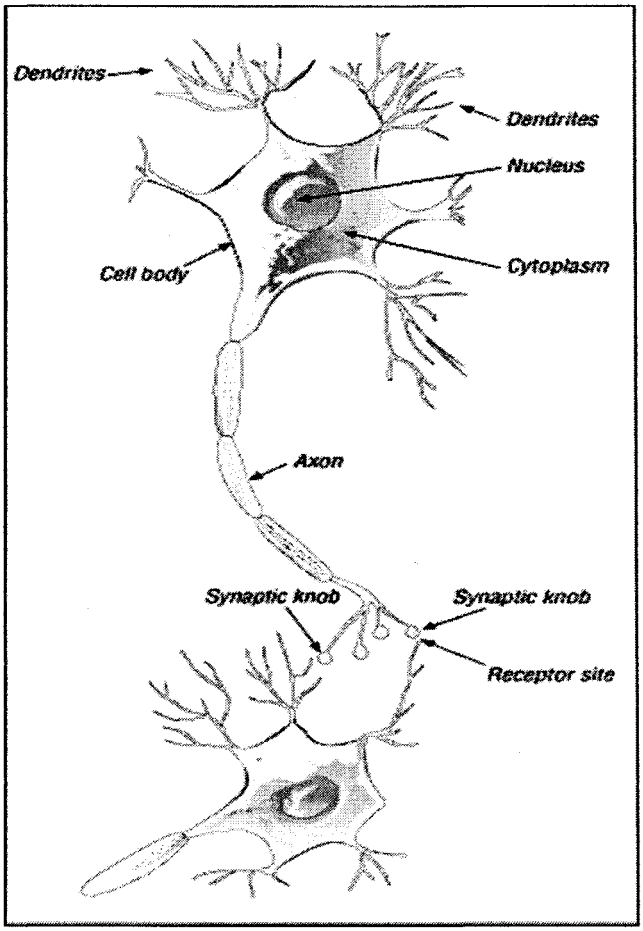

Figure 1.5: Schematic diagram of cell to cell contact via synapses (Kitazawa *et al.*, 1998). This contact has been speculated to facilitate BDV antigen distribution in CNS via cell to cell dissemination through synaptic contacts between neurons (Bajramovic *et al.*, 2003).

Thus BDV replicates in cells in these areas of the brain i.e. neurons, Purkinje cells and ventral motor neurons of the spinal cord, astrocytes, oligodendroglia, ependymal cells and Schwann cells. However, BDV is not restricted to the CNS and can also be found in many other parts of the body including peripheral blood mononuclear cells,

kidney, thymus and bone marrow during persistent infection. Furthermore, Madin-Darby canine kidney (MDCK) and Vero cells, which are not derived from the CNS are also susceptible to virus infection and readily become persistently infected (Stitz, & Rott, 1999; Gosztonyi & Ludwig, 1995). The replication cycle takes about 24 hours to be completed and, as demonstrated by the relatively low replication rate of BDV (Stitz and Rott, 1999), only a little virus if any is released from infected cells.

1.1.2.4. Effects of BDV on the central nervous system

Neurotransmitters and modulators are one of the central means for communication among neurons in the central nervous system. Various neurotransmitter pathways have been shown to be involved in the pathogenesis of Borna disease although the specific mechanisms of this involvement are not fully established (Lipkin *et al.*, 1988).

It is also uncertain which neurotransmitter pathways are affected in response to direct viral action and which are influenced by the more generalised inflammatory response in the brain. Swelling and damage to the cortical cholinergic axons is found in animals prior to the development of encephalitis and the progressive deterioration of this system is believed to parallel the development and advance of BDV-induced encephalitis. In general, mediators strongly implicated in Borna disease are glutamate, cholecystokinin, decaroxylaste, somatostatin, and the dopamine system,

which has already been implicated in other psychiatric conditions (Lopez-Figueroa *et al.,* 2004; Wassef *et al.,* 2003). For example, there are reports on Alzheimer's disease where the basis of neurochemical sensorimotor defects and memory impairment was shown to be acetylcholine and it has thus been suggested that this molecule is one of the most important neurotransmitters for integration of sensory and motor information, learning and memory (Kesner, 1988). Reports on BDV infection also show that such defects, as well as dementia, occur. The disruption of the ability of astrocytes to prevent glutamate-mediated neurotoxicity may contribute to BDV-induced CNS dysfunction in the absence of encephalitis by cortical cholinergic denervation (Gies *et al.,* 1998).

1.1.2.5. Clinical manifestation of BDV infection

The pathogenesis of BDV is complex and reports indicate that natural and experimental infection lead to different pathological events. Moreover, different animal species can present different clinical manifestations depending on age and route of infection.

During natural infection the incubation period ranges from two weeks to several months with an average of two to three months depending on the host status, i.e.

species, genetic background, age, immune status and virus strain (Durrwald & Ludwig, 1997; Katz *et al.,* 1998; Carbone *et al.,* 1987).

Following this incubation period some animals present alterations in behaviour, disturbance in learning and memory, obesity, disturbances in fertility, ataxia, blindness, and also in late stages of the disease after centrifugal migration of the virus along the optic nerve, retinopathy and visual impairment, paralysis and finally death (Rott & Becht, 1995; Hagwiara *et al.,* 1997; Herden *et al.,* 2000). In some cases spontaneous recovery can be observed despite a persistent infection of the CNS with virus, although at times recurrence of disease can also occur (Staeheli *et al.,* 2000; Mayr & Danner, 1974). However, some infected animals may be asymptomatic and appear uninfected (Nakamura *et al.,* 1996; Kao *et al.,* 1993).

The encephalitis observed in infected animals is the result of the host immune response to viral infection. In immunocompetent animals inflammation results from the infiltration of $CD4^+$ and $CD8^+$ T cells, although the exact mechanism of $CD8^+$ T cell pathogenesis is not fully understood (Blizer *et al.,* 1995). The immunopathogenic process is believed to be mediated by $CD8^+$ T cells, while $CD4^+$ T cells play a helper role with T-cells recognizing a specific epitope on viral nucleoprotein p40. Thus the immune response to viral antigens does not elicit a protective immunity but rather an immunopathologic reaction in which T cells play an important role (Narayan *et al.,* 1983).

Animals chronically infected with BDV have high levels of viral antigen and RNA in the CNS. However, only extremely low levels of enveloped infectious virions are detected, and viral budding has not yet been observed in BDV infected tissues and cells (Gosztonyi & Ludwig, 1995).

In experimental infections animals such as rats have been used to show that inflammation of nerve cells in the brain are caused by the immune system as immunocompromised animals (experimentally immunocompromised, or with removed thymus) do not develop clinical signs of the disease despite productive BDV replication in the CNS (Richt *et al.,* 1997). Such animals do not experience any interference with their vital functions showing that the disease itself is the result of a virus-induced cell-mediated response by the host. Similarly, a recent study showed that immunosuppressed humans have a decreased risk of clinical disease and there is no clinical indication that BDV can act as an opportunistic pathogenic agent (Cotto *et al.,* 2003).

A number of animal species are less susceptible to BDV (chickens, monkeys, cattle, and tree shrews) while others are more susceptible (rabbits, Lewis rats, and guinea pigs). A further, third group, of animals (pigeons and dogs) can be persistently infected with BDV but never develop pathological consequences (Richt *et al.,* 1992; Rott & Becht, 1995; Becht & Richt, 1996; Sprankel *et al.,* 1978). Similar to natural infection BDV can also experimentally infect a variety of animals; however incubation periods, clinical signs, and severity of the disease depend on the animal

species, transmission route and the virus variants. For example, tree shrews and rhesus monkeys show different behavioural symptoms after infection. Tree shrews show altered social and sexual behaviour manifested as abnormal dominance relationship and failure to mate (Sprankel *et al.,* 1978) whilst rhesus monkeys initially can be hyperactive and subsequently become apathetic and hypokinetic (Stitz *et al.,* 1980).

After adult rats are infected intracerebrally or intranasally productive virus replication is found in the entire CNS and usually causes a persistent infection with continuous productive replication in the brain and spinal cord (Richt *et al.,* 1997b). The animal develops an encephalomyelitis with infiltrating $CD4^+$ and $CD8^+$ T cells and macrophages (Bilzer *et al.,* 1995; Deschl *et al.,* 1990). During the acute phase $CD8^+$ T cells significantly contribute to the destruction of virus infected brain cells and the degenerative encephalopathy that follows. Once the animal becomes chronically infected, atrophy of the cortical brain is observed due to these same T cells. However, it was also observed that if the animals were injected with BDV-specific $CD4^+$ T cells prior to becoming infected with BDV, this specific $CD4^+$ T cells would induce the production of BDV-specific $CD8^+$ cells. In such conditions, BDV-specific $CD8^+$ cells would have a major function of eliminating BDV and significantly reducing cell damage (Planz *et al.,* 1998; Bilzer & Stitz, 1996).

These animals (rats) present with the following symptoms: hyperactivity and exaggerated startle reactions, in the acute phase. It is suggested that this is due to

viral gene products in the limbic system (Narayan *et al.*, 1983; Carbone *et al.*, 1987). The infection becomes persistent and adult animals show stereotyped motor behaviour, dyskinesias and dystonias associated with distinct changes in the CNS dopamine system as well as increased activity and cachexia (Narayan *et al.*, 1983; Solbrig *et al.*, 1994; 1995; 1996a; 1996b; Planz *et al.*, 1998).

In infected neonates the histoimmunophatology differs from the adult. Neonatal rats develop a persistent tolerant infection characterized by a lack of inflammation in the nervous system and no overt signs of a neurological disorder (Bautista *et al.*, 1994; Herzog *et al.*, 1984). Despite the absence of encephalitis and clinical signs of classic BD, these neonates exhibit stunted growth, hyperactivity, subtle learning disturbances and altered taste preferences. Moreover, they do not mount a cellular immune response to the virus (Dittrich *et al.*, 1989; Carbone *et al.*, 1991). In addition the virus is present throughout the whole organism and BDV-specific antigen can be found in parenchymal cells of numerous organs and infectious virus can also found in excretions (Herzog *et al.*, 1984). Thus the age of rats at the time of infection seems to affect the capacity of host cells to support virus replication and, therefore, to control the spread of BDV.

Overall experimental infection resembles the natural infection in animals and the pathogenesis of BD in BDV-infected animals indicates that BD is also an immunopathological disease. Immunosuppression and/or splenectomy affect the severity of clinical signs or prevent disease.

1.2. Overview of human psychiatric diseases

1.2.1. The human brain structure

The brain encloses the central nervous system (CNS) including the nerves of the spine. The CNS has a complex and not fully understood job of receiving, processing and signalling via a range of chemical and electrical impulses (figure 1.6). The two types of cells found in the brain are neurons (information transmitters) and glial cells (controls the uptake of nutrients and other chemicals, repair and protect the brain).

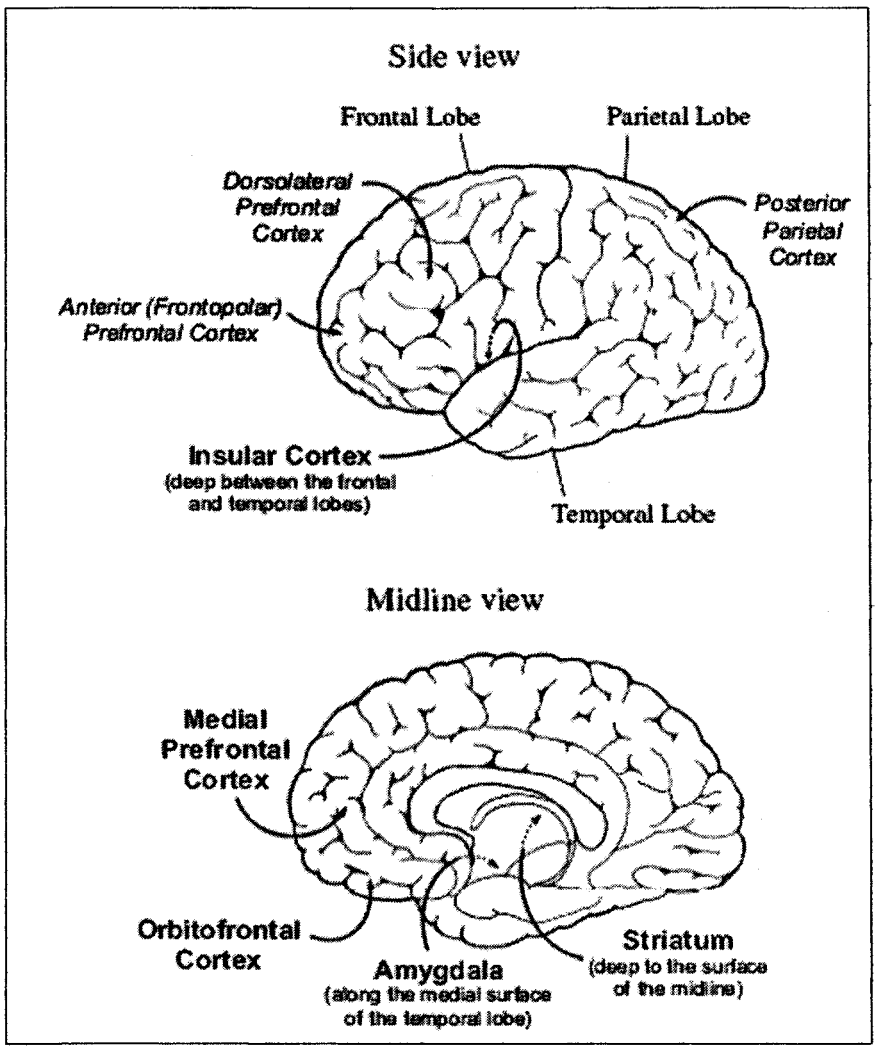

Figure 1.6: Human cerebrum: Lateral (side) and medial (midline) views, identifying areas critically associated with decision making. Areas in bold have consistently been associated with emotional processing, while areas in italics have consistently been associated with higher level cognitive processes (extracted from Cohen, 2005) Note that the hippocampus is a structure adjacent to the amygdala.

The brain can be divided into three parts (figure 1.6) as follows:

1) The forebrain

This includes the hypothalamus (controls hunger, thirst, sexual behaviour, body temperature and motivation), thalamus (the relay centre for sense receptors), the cerebral cortex (higher-order thinking and language) and the corpus callosum (band of fibers that connects the two hemispheres) (Coughlan & Warrinoton 1978).

2) The midbrain

Only contains the reticular formation

3) The hindbrain

This region includes the cerebellum (controls balance, fine movement and muscle tone), the pons (controls the sleep-wake cycle) and the medulla (controls breathing, heart rate and blood pressure).

The CNS controls body functions, produce thought, emotions and memories. However, the knowledge of which parts of the brain are affected during psychiatric disease is very limited, although differences in pre-frontal, anterior paralimbic areas

and amygdale have been suggested (Lane *et al.,* 1997; Bradley, 2001; McGaugh *et al.,* 1993).

1.2.2. Classification of psychiatric disorders

Psychiatric disorders are groups of conditions related to brain function, where the affected individuals present a specific profile that includes self-centeredness, immaturity, excessive dependency, anxiety, tendencies to deny illness, histrionic behaviour, and poor tolerance of frustration or courage, resilience, conscientiousness, modesty, and adaptability (Soares & Mann, 1997). These individuals can have a history of repetitive behaviour during stress--whether distress is expressed in physical symptoms (i.e. headache, abdominal pain), psychological symptoms (i.e. phobic behaviour, depression), or social behaviour (i.e. withdrawal, rebelliousness; Soares & Mann, 1997).

The causes and pathogenesis of psychiatric disorders have yet to be determined with certainty, but are thought to have a multitude of origins. These can be related to biological factors (i.e. genetic, circadian rhythms, brain structure, electrical magnetic activity, chemistry and immune-system), environmental factors (i.e. stress, gender and culture), drug related (i.e. steroids, sedatives) and infectious agents (i.e. BDV, cytomegalovirus, herpes simplex virus) (Salvatore *et al.,* 1997; Carpenter *et al.,*

2000; Tamminga, 1999; Sanacora *et al.*, 1999; Dickerson *et al.*, 2005; Buka *et al.*, 2002).

Psychiatric disorders can be divided into different categories (figure 1.7) according to a selective criterion. The diagnosis is given by taking into account inclusion and exclusion criteria for the number of signs present and determined by the Diagnostic and Statistical Manual of the Psychiatric Association (DSM-IV; revised fourth edition).

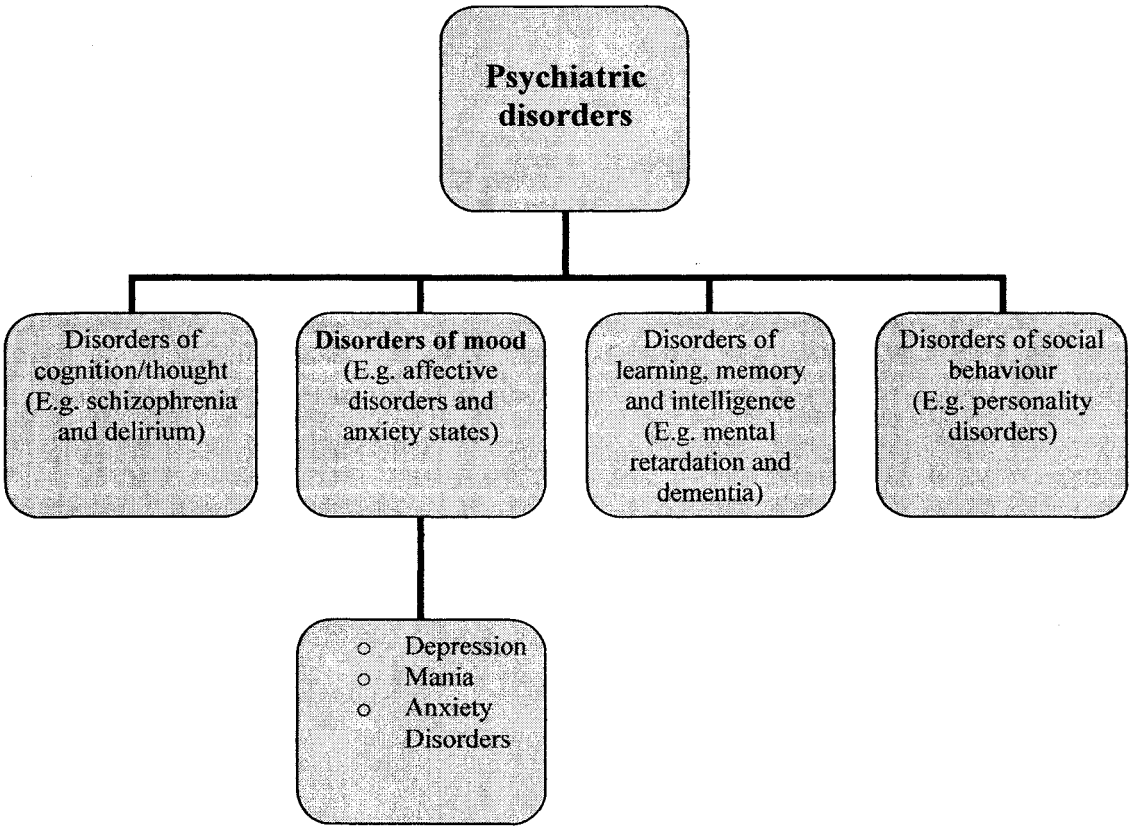

Figure 1.7: Psychiatric disorders classification. They were grouped into 4 main areas and only the sub-classification of the mood disorder group is presented.

1.2.2.1. Sub-classification of mood disorders (MD)

Mood disorders can be identified by lesions of sub genual PFC and can lead to difficulties in expressing emotions, abnormal autonomic responses to emotionally arousing stimuli and to a compromised ability to reason and make intelligent rational decisions. They are affective disorders mostly found in higher socioeconomic groups and are a group of heterogeneous, typically recurrent illnesses that include unipolar (depressive) and bipolar (manic-depressive) disorders. They are characterised by pervasive mood disturbances, psychomotor dysfunction, and vegetative symptoms (Torrey 1999).

In general mood disorders are classified as:

- Major Depressive Disorder (2+ weeks of depressed mood or loss of interest plus at least 4 additional symptoms of depression).
- Dysthymic Disorder (2 + years of depressed mood plus < 4 additional symptoms of depression)
- Bipolar I Disorder (1+ Manic or Mixed Episodes accompanied by a Major Depressive Episode and requiring hospitalization).

- Bipolar II Disorder (1+ Major Depressive Episodes accompanied by a Manic Episode or illness with hypomania).
- Cyclothymic Disorder (2 + years of frequent periods of manic symptoms falling short of the criteria for a Manic Episode and depressive symptoms falling short of the criteria for a Major Depressive Episode).

Depression is a very common mental condition characterised by feelings of sadness, loneliness, despair, low self-esteem, and self-reproach (Lane *et al.,* 1997). Depression can be followed by psychomotor retardation or at times agitation, withdrawal from interpersonal contact, and vegetative symptoms such as insomnia and anorexia (Birketvedt *et al.,* 1999). Depression (unipolar disorder) in its full bloom manifests as a major depressive disorder, with an episodic course and varying degrees of residual manifestations between episodes. On the other hand, bipolar disorder (or manic depression) is associated with moods that swing from periods of mania (mood elevation, including exaggerated euphoria, irritability, or both) to episodes of depression. These patients often have multiple relapses that may lead to unemployment, marital problems, alcohol abuse, and suicide (Carpenter *et al.,* 2000; Felitti *et al.,* 1998; Jamison, 2000).

Although both women and males are similarly affected, a higher number of women are diagnosed with mixed states, and cyclothymia. According to DSM-IV (1994), the lifetime prevalence for bipolar I is 0.4–1.6 % and for bipolar II 0.5 %. However,

males are diagnosed with bipolar disorders much earlier in life and are reported to have more severe presentations of the disease (Lewinsohn *et al.,* 1994). This is supported by reports on the early onset of bipolar disorder where patients were diagnosed as children (McGlasham, 1988) and adolescents tend to have a more severe form and to have more complications (i.e. behaviour disorders, substance abuse, paranoia) as well as less frequent remissions (Lewinshon *et al.,* 1994). Women tend to have an adult –onset (age 40). This contrasts sharply with lifetime prevalence rates shown in the same manual for major depression (single or recurrent) as 5 – 12 % for male and 10 – 25 % for female patients (Dunner *et al.*, 1976).

Patients with bipolar disorders exhibit different but increasing symptoms over the years with infrequent and unpredictable episodes of depressive and manic phases (average of 8 to 10 manic or depressive episodes over a lifetime) as well as severity (Akiskal *et al.,* 2000; Sato *et al.,* 2003). This is the typical pattern for bipolar patients and is referred as *non rapid cycling* where, patients can experience single episodes of depression or mania, however they can also present mixed state, where both mania and depression are present. The other less common cycle pattern observed in bipolar patients (~15 %) is referred as *Rapid Cycling* where patients experience a temporary but complicated phase of depression and mania. These patients present switch from a manic to a depressive episode at least four times a year (in severe cases, several cycles a day; Post *et al.,* 2003).

Bipolar disorders, as with any other psychiatric condition, tend to occur more frequently in people born in the winter and episodes of mania and depression tend not to occur at the same time of the year. For instance, patients exhibit most frequently episodes of mania in the summer while depressive episodes are more observed during early winter to early spring. Similarly, they do not have a clear cause but risk factors such as genetics, other neurologic conditions (i.e. attention deficit hyperactivity disorder (ADHD), brain structure and chemistry, personality traits (lack of persistence, avoid harmful situations, and be dependent on rewards), higher socioeconomic status, early loss of a parent, seasonal variation and infectious agents are all implicated (Benes and Berretta, 2001; Torrey *et al.,* 1997).

1.2.2.2. Neuropathogenesis of mood disorders

Inflammation, decrease and death of brain cells as well as hyperactivity of systems produce functional and neuropathological changes in the cerebral cortex of depressed patients (Harrison, 2002) leading to interference in chemical structure observed in bipolar patients. These changes lead to the neuro-chemical theories of psychiatric conditions in particular the increased activity of the hypothalamic-pituitary-adrenal (HPA) axis. Disturbances of neurotransmitters such as catecholamine, serotonin, glutamate, γ-aminobutyric acid (GABA), neuropeptide, signal transduction and developmental/synaptic systems that are involved in the pathogenesis of bipolar disorders and can lead to depression are also disturbed during BDV infection

(Carlsson *et al.,* 2004; López-Figueroa *et al.,* 2004; Tamminga 1999; Wassef *et al.,* 2003; De Wied & Sigling 2002; Gould and Manji 2002; Lipska & Weinberger 2002; Honer &Young 2003).

Considerable attention has been given to GABAergic interneuron, a potent neurochemical, and their neurochemical marker which provides glutamine to neurones (Taylor *et al.,* 2003). Several studies analysing the involvement of the GABA system reported that there is a decrease in neurochemical markers (reelin, parvalbumin, GAD_{65}, synaptophysin, glutamic acid decarboxylase (GAD) and GABA) after infection with influenza virus, human immunodeficiency virus, lymphocyte choriomeningitis, Coxsackie B4 virus, Venezuelan equine encephalomyelitis virus , and BDV(Cochran *et al.,* 2003; Fatemi *et al.,* 1999; Masliah *et al.,* 1992a, 1992b; Bonila *et al.,* 1980; Gonzalez-Dunia *et al.,* 2000). The mechanisms underlying this reduced cortical GABA are still unknown and might be also related to a number of metabolic disturbances (Sanacora *et al.,* 1999). Stress which has been implicated in bipolar disorders does not appear to be an influence as stress is known to decrease the levels of glutamine on GABA system (Benes and Berretta 2001).

1.2.2.3. Overview of mood disorder treatment

The treatment of bipolar disorder includes mood stabilisers, anxiolytics, anticonvulsants, antidepressants, antipsychotics and antivirals. The treatment alleviates symptoms caused by the electrical, physical, immunological and chemical effects of the brain (Wang & Ketter, 2003; Lieb *et al.,* 2002). Surprisingly some antidepressants (i.e. amantadine and lithium) have antiviral effects and seem to be effective in treating both bipolar disorders and BDV (Ferszt *et al.,* 1999; Quitkin *et al.,* 1981; Berk *et al.,* 2001; Compton & Nemeroff, 2000; Young *et al.,* 2000; Walden *et al.,* 1996).

1.3. Borna disease virus and human mood disorder

The observations that BDV can infect a wide range of animals and the observation that behavioural disturbances in animals infected with BDV resembles some aspects of human neuropsychiatric diseases suggests that BDV may affect humans (Hatalski *et al.,* 1997).

In natural infections BDV preferentially behaves as a neurotropic virus, although it may latently and/or persistently infect cells of the mononuclear phagocytic system (Carbone *et al.,* 1993). The virus has a preference for cells of the limbic system includes the amygdala and hippocampus), which is the most delicate and sensitive

old area of the mammalian brain and is involved in the control of mood, behaviour, and memory (figure 1.7).

In many infected individuals BDV appears perfectly adapted to persist as commensal organism inside the system throughout the lifetime of that organism showing no cytopathogenicity or disturbance of vital cell functions (Carbone *et al.*, 2001). Thus these individuals present with no symptoms and remain undiagnosed (Ludwig & Bode, 1997). However, a poorly defined mechanism responsible for altering central functions can be switched and lead to a degenerative disorder and the lifelong persistence of BDV is expected to lead to periods of long latency and short activation leading to episodes of distinct, more or less severe disturbances of information processing and behavioural and mood alterations (Bautist *et al.*, 1994; Dietrich *et al.*, 1989). BDV appears to have a specific affinity for aspartate and glutamate receptors in the hippocampal formation and has been reported to directly induce an imbalance in these transmitter system interactions (Dietrich *et al.*, 1998). Similar to mood disorders, the affected brain has significant alterations in the regional tissue content of serotonergic (serotonin; 5-HT) or dopaminergic neurotransmission resulting in a compromised axonal transport and/or release of 5-HT (sub-section 1.2.2.4/1.3.2.2; Dietz *et al.*, 2004).

The symptoms of BDV infection in man may not be exactly the same as that seen in animals and it has been suggested that humans may not have the typical symptoms of viral encephalitis such as fever or changes in mental alertness. Instead, they may

express signs of psychiatric disease, such as depression, mania, anxiety, cognitive disorders, tardive dyskinesia, social dysfunction, eating disorders, and idiopathic seizures (Pletnikov *et al.,* 1999a; Rubin *et al.,* 1999; Solbrig *et al.,* 1994). Additionally it has been suggested that transplacental infection is possible in humans. The clinical signs that an individual is infected vertically are similar to those individuals infected during the first 3 years of their life. Their signs are much more prominent and these subjects may move on to have an autistic spectrum of disorders, with abnormal social interactions, chronic anxiety, cognitive deficits, and abnormal development of the cerebellum and hippocampus (Bautista *et al.,* 1995; Carbone *et al.,* 1993; Dittrich *et al.,* 1989; Eisenman *et al.,* 1999; Pletnikov *et al.,* 1999b, 2002; Rubin *et al.,* 1995).

The suspicion of the involvement of BDV with human neuropsychiatric diseases and with a number of other conditions is suggested by finding BDV RNA, BDV proteins, anti-BDV antibody and/or infectious virus in the blood, cerebro-spinal-fluid (CSF), and/or brains of patients with chronic fatigue syndrome (Kitani *et al.,* 1996; Nakaya *et al.,* 1996), HIV-infected patients (Auwanit *et al.,* 1996), patients with schizophrenia or with deficit syndrome schizophrenia subtype (Chen *et al.,* 1999a; Iwahashi *et al.,* 1997, 1998; Nakamura *et al.,* 2000), patients with multiple sclerosis and/or depression (Deuschle *et al.,* 1998; Yamaguchi *et al.,* 1999) and patients with dementia and hippocampal degeneration (de la Torre *et al.,* 1996). BDV has also been isolated from brain tissue of some apparently healthy people (Haga *et al.,* 1997) and BDV RNA, BDV proteins, anti-BDV antibody have also been found in blood

donors (Kishi *et al.*, 1995; Thomas *et al.*, 2005). However definitive evidence that BDV is the cause of any of these conditions has yet to be established although seroepidemiological studies have consistently shown an increased BDV seroprevalence and higher BDV RNA prevalence in peripheral blood mononuclear cells of neuropsychiatric patients as compared to healthy controls (Bode 1995; Hatalski *et al.*, 1997; Lipkin *et al.*, 1995; Richt *et al.*, 1997b). There are a number of reported cases where 10 to 50 % of neuropsychiatric patients are positive for BDV as shown by serology as opposed to healthy blood donors (0 to 4.6 % BDV positive) (Bode *et al.*, 1995; *Kishi et al.*, 1995; Nakaya *et al.*, 1996; Sauder *et al.*, 1996). BDV antigen and RNA have also been detected in human brain samples collected at autopsy from individuals with a history of mental disorders (de la Torre *et al.*, 1996; Haga *et al.*, 1997; Salvatore *et al.*, 1997), as well as in clinical samples (i.e. glioblastomas) (Nakaya *et al.*, 1996).

Together these findings suggest that BDV can infect humans and persist in the CNS and that BDV is also associated with certain mental disorders although these epidemiological and clinical studies remain controversial.

In general it is accepted that depression is associated with disturbances of the neuroendocrine functions and these disturbances may have a viral cause and treatment involves regulating the neurological factors that are unbalanced to restore the growth and survival of neurons (figure 1.8).

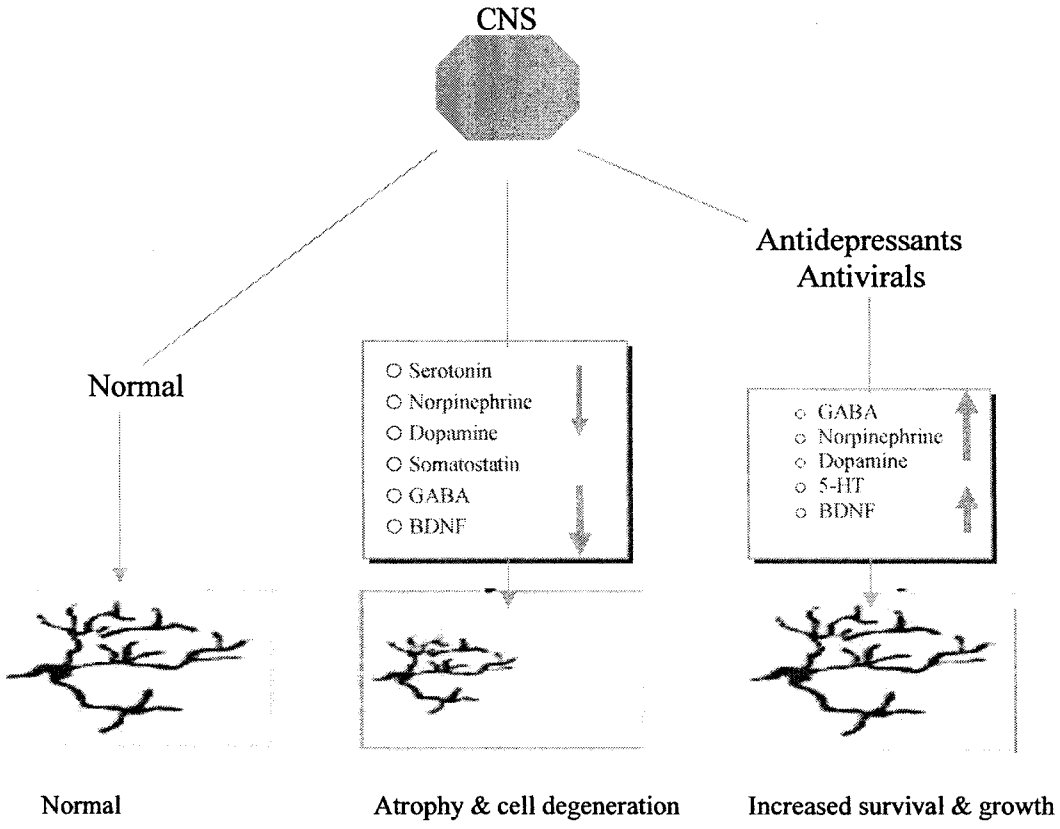

Figure 1.8: Association of decreased neuroendocrine functions and antidepressants

NB: Other neurotransmitters are also disturbed but not shown on the diagram.

Experimental treatment of BDV infection is also controversial. Studies have demonstrated that certain drugs such as amantadine sulphate and ribavirin, used as antidepressive and antiviral agents, can also be used to treat BDV infection (Bode *et al.*, 1997; Hallensleben *et al.*, 1998).

Amantadine is a dopaminergic, noradrenergic and serotonergic substance that blocks monoaminoxidase A and NMDA receptors and seems to raise beta-endorphin/beta-lipotropin levels (Huber *et al.,* 1999; Bode *et al.,* 1997). Although amantadine acts through several pharmacological mechanisms its modes of action in treating depression is still not well defined (Dietrich *et al.,* 2000a). However, it has been extensively used in the treatment and/ or prophylaxis of influenza A virus, Parkinson's disease, traumatic head injury, dementia, multiple sclerosis, bipolar disorder and cocaine withdrawal among others (Huber *et al.,* 1999; Dietrich *et al.,* 2000b; Thompson *et al.,* 2003; Schapira, 1999; Kampman *et al.,* 2000).

Ribavirin, a guanosine analogue, has never been tested in humans with evidence of BDV infection although it seems to inhibit BDV transcription in rats (Carbone, 2001; Jordan *et al.,* 1999). The suggested mechanism for ribavirin includes the reduction of the intracellular GTP pool and this in turn will inhibit the transcription and capping of BDV mRNAs and thus is a potential drug for BDV treatment (Jordan *et al.,* 1999). However, both amantadine and ribavirin seem only to be effective in certain types of cells (i.e. oligodendrocytes, glia cells) and with certain primary virus isolates (i.e strains V and He/80) (Hallensleben *et al.,* 1998; Jordan *et al.,* 1999; Bode *et al.,* 1997).

Other inhibitors of cellular and DNA polymerase used in the treatment of a number of diseases (i.e Leukemias) have been reported to have an effect on the inhibition of BDV RNA and protein synthesis (Bajramovic *et al.,* 2002). An example of this is 1-ß-D-arabinofuranosylcytosine (Ara-C) a nucleoside analogue that has been experimentally used as an alternative drug to ribarin and this may also potentially be used to treat humans with BDV infection (Furth & Cohen, 1968; Bajramovic *et al.,* 2002).

Little has been reported on the role of lithium a first line mood stabilizer drug used in the treatment of bipolar disorder that also seems to have an antiviral effect. Lithium normalises the systemic immune activation associated with depression by stimulating the release of the neurotransmitter glutamate (Manji *et al.,* 1999, Moore *at al.,* 2000; Kopnisky *et al.,* 2003).

BDV infection in humans and its relationship to psychiatric conditions is still highly controversial and this study will look at BDV infection by detection of viral markers such as antibodies, antigens and circulating immune complexes (CIC) in both patients and healthy individuals. The presence and level of antigenemia and CICs will give data as to the relationship between BDV and human bipolar disorders.

1.4. Diagnostic Tests for BDV Infection

The identification and diagnosis of BD disease, its biological characteristics and infectivity patterns is unclear possibly due to the low viral productivity of BDV and the tight association of BDV with plasma membranes. However progress towards developing new diagnostic reagents, primers and probes are making improvements in the diagnosis of this virus (Hatalski *et al.,* 1997; Sierra-Honigmann *et al.,* 1993).

At present numerous tests are used for BDV detection and diagnosis. They either detect the virus or the host immune response to the virus. These tests include assays for anti-BDV antibody, and BDV proteins (i.e. radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA)), RT-PCR assays for BDV RNA and *in vitro* or *in vivo* assays for infectious BDV particles (infected cells, infected cell extracts, and recombinant proteins produced in prokaryotic or baculovirus systems) (Saunder *et al.,*1996; Staeheli *et al.,* 2000). These can be grouped into four main categories:

1. Biological/virological methods to measure virus infectivity
2. Serological methods for the detection of host antibody responses to the virus and for detection of virus antigens (proteins or glycoproteins)
3. Molecular methods for detection of viral nucleic acids or genes (cDNA or RNA)
4. Imaging techniques for the direct visualisation of virus particles

Despite the fact that a variety of these tests are used for the detection of BDV, there are no universally accepted standards for diagnosis of human BDV infection, possibly due to limited data available on inter-assay variations and discrepancies between investigators reflected by differences in clinical populations, assay sensitivity and specificity, viral titres or other (Carbone, 2001).

Based on observations that the use of a single test for BDV epidemiological studies may not provide detailed enough information a range of diagnostic tools for the detection of viruses and virus components in biological samples were selected for this study. These included molecular, immunochemical, serological and virological techniques to identify BDV-infected subjects.

1.5.1. Virological/biological diagnosis for BDV detection and measurements

In general virological/biological diagnosis includes those methods used for virus isolation (e.g. cell culture), direct visualisation of virus particles (electron microscopy) and for detection of virus proteins (e.g. fluorescent- focus assay, endpoint dilution assay, virus neutralization, viral serotypes, immunostaining, and flow cytometry).

It is not easy to recover BDV from body fluids, as BDV is largely a cell-associated virus, thus the isolation of infectious virus is achieved by inoculating tissue homogenates into cell culture or by *in vivo* testing (i.e. animal infectivity assays). Co-cultivation of BDV strains is the gold standard diagnostic technique and is a sensitive method that is required to show BDV infectivity and determine susceptibility of strains to antiviral agents. In general, the virus titre in the inoculated test material determines the time of BDV detection in the homogenates and infectivity can vary from 24 hours if the material has a high-titre to several months if the material has a low-titre. Investigators have successfully isolated BDV from both human and animal brain and the most usual cell lines for recovery of infectious BDV include primary neural cells from either rat or rabbit and neurological cell lines such as C6 rat glioma or OL human oligodendroglial cells (Bode *et al.,* 1996; Carbone *et al.,* 1993). A number of investigators have used brain homogenates from the hippocampus, rhinencephalon and spinal cord inoculated into human oligodendroglial cell lines, young rabbit brain cells and Vero cells. Human BDV has normally been isolated from brain tissue collected at autopsy from BDV-seropositive psychiatric patients with inflammatory changes in the hippocampus.

The expression of viral antigen is normally monitored by immunofluorescence (IF), RT-PCR (large array of primer pairs), northern blot hybridization, immunohistochemistry and Western blot analysis (Nakamura *et al.,* 2000; Nowotny *et al.,* 2000).

As with any other technique co-cultivation also presents drawbacks and it is not always possible to recover the infectious virus. This may be due to low BDV replication levels in some species, the relative restricted replication preference and viral titre (Carbone, 2001; Bode *et al.*, 1996).

In this study BDV was isolated from ultra-sonicated peripheral mononuclear cells (PBMCs) homogenates obtained from a BDV-positive patient inoculated into a human oligodendroglia (OL) cell line. The cell culture allowed propagation of human BDV isolate and infectivity was confirmed by serological and molecular techniques, which are referred to in more detail in their specific subsections.

1.5.2. Serological diagnosis for anti-BDV antibody and BDV proteins

Serodiagnosis is a useful tool to determine exposure to and infection with BDV. Methods include immunoassays, immunocytochemistry, and Western blotting (introduced with isolation). These tests are used to either detect viral proteins or presence of humoral immune responses against the virus. These may be designed to identify virus-specific IgM (for diagnosing acute infection) or virus-specific IgG (for screening, for diagnosing chronic infection or prior resolved infection, and for assessing immune status). Methods that measure anti-viral antibodies indicate exposure to BDV rather than the presence of the virus (i.e., active viremia). These

tests are relatively simple to perform thus they may be used for screening purpose but they may be relatively non-specific (Waltrip *et al.,* 1995).

Three serological methods have been used to detect antibodies to BDV and BDV antigens in human sera: indirect immunofluorescence (IFA), Western blot and ELISA. The main advantage of serological methods for diagnostic studies is that as a result of immunological memory serum antibodies allow the detection of long lasting antibodies as well as resolved viral infections. Unfortunately for the diagnosis of BDV infections in animals or humans serological methods are not perfect as BDV-specific antibodies are usually not that high e.g. IFA titres are frequently below 1:40 in naturally infected animals with full-blown Borna disease (Herzog *et al.,* 1984).

1.5.2.1. Immunofluorescence assay (IFA)

The immunofluorescence assay (IFA) was the first diagnostic test used for BDV diagnosis and was designed to detect anti-BDV antibodies (Rott & Becht, 1995; Amsterdam *et al.,* 1985). IFA has been reported to be one of the most reliable and sensitive methods among serological assays for detection of antibodies (Billich *et al.,* 2002; Allmang *et al.,* 2001) although as with any other diagnostic tool is subject to variation. Staeheli *et al.,* (2000) gave a description on several reports looking for anti BDV antibodies in horses and sheep and there was great variation in these studies.

For example one study showed 100 % seropositive animals while in others the percentage varied between 41 % and 73 % (Bilzer *et al.*, 1996; Katz *et al.*, 1998). A high prevalence of BDV infection in humans, as shown by IFA, has also been reported and this varies from 0 % to 50 % (appendix V). Thus it has been concluded that IFA provides variable results across laboratories probably due to differences in the cell systems used and titres of BDV antibodies.

In the IFA technique, human serum is overlaid onto a slide covered with fixed BDV-infected cells. Anti-BDV antibodies in the serum will then bind to viral antigens expressed by the cells (figure 1.9). BDV-infected Madin-Darby canine kidney (MDCK) cells (Herzog & Rott, 1980) or C6 (glial cells) (Carbone, 2001) cells are most commonly used in the IFA however, in this study YRS cells was used.

A monoclonal mouse anti-BDV antibody mixed with the test serum sample with a second-colour fluorescently labelled anti-mouse IgG are added with the fluorescently labelled anti-species IgG (Bode *et al.*, 1992; Waltrip *et al.*, 1995). Typically when testing human sera a FITC labelled anti-human IgG antibody will be used to detect anti-BDV antibody in the test sample and a TRITC labelled anti-mouse antibody will be used to show that the signal for the test sera co-localises the cell with the monoclonal anti-BDV antibody. This confirms that the antibody in the test sera is specific for BDV rather than a non-specific nuclear antibody.

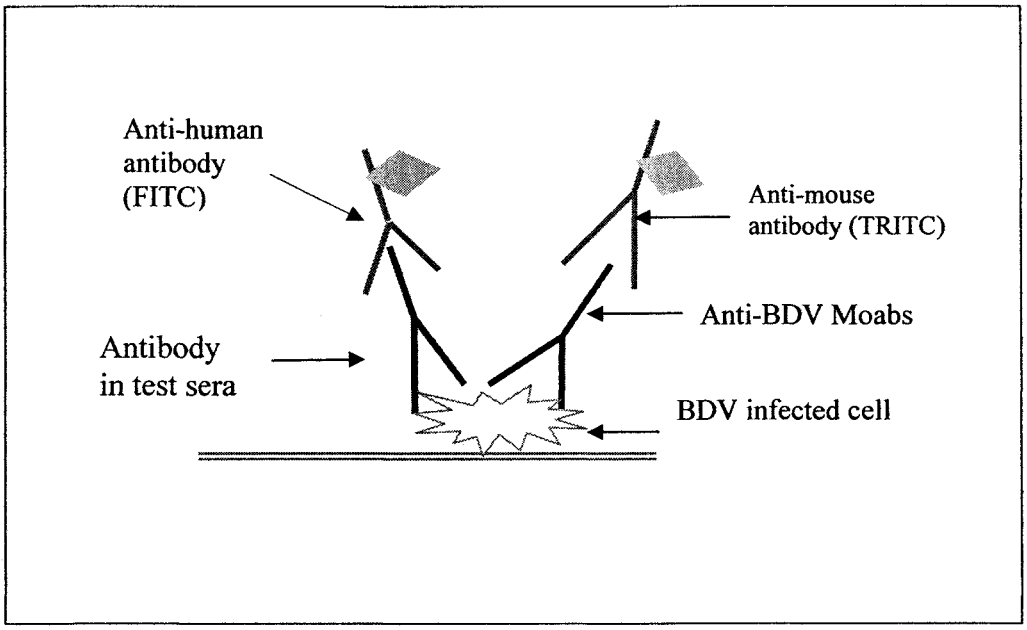

Figure 1.9: The Immunofluorescence Test. The diagram illustrates the specific binding of antibodies to antigen in the reaction. Moabs = monoclonal antibodies. Note that, the presence of the BDV antibody iin the test sera is detected by the FITC labelled anti-species antibody, which fluoresces green, whilst the presence of BDV in the BDV infected cell is confirmed by the TRITC labelled anti-mouse antibody (fluoresces red), which will bind to the anti-BDV moabs.

For the control of false positive results test samples are tested on uninfected cells and a positive control would normally be polyclonal anti-BDV serum produced in experimentally infected animals.

1.5.2.2. Confirmatory diagnosis of BDV infection by Western blot assay (WB)

Similar to the IFA, western blot assay detect human antibody by its binding to virus antigens. In WB, virus antigens are separated by SDS PAGE electrophoresis and then transferred to a specialised blotting membrane (nitrocellulose). Strips of the blot are incubated in human serum and washed. Binding of specific anti-BDV antibodies is detected by a secondary enzyme-labelled anti-human IgG antibody and is indicated by enzymatic reaction which results in a visible insoluble dye or a light signal which can be captured on X-ray film or image equipment. The image can be evaluated qualitatively by eye. Blots include molecular weight markers to confirm the location of the known sizes of BDV proteins and positive and negative controls obtained from infected animals to confirm the appropriate performance of the assay.

Previous serological studies of BDV in man have shown the prevalence of anti-BDV antibodies in psychiatric patients varies from 0 % to 45 % in different laboratories by this method (Chen *et al.*, 1999a; Iwahashi *et al.*, 1998; Kishi *et al.*, 1995a, 1995b; Rott *et al.*, 1991; Saunder *et al.*, 1996; Tsuji *et al.*, 2000; Waltrip *et al.*, 1995). The WB appears to give lower seroprevalence of BDV when compared to that seen by IFA (highest 50 %) and ELISA (highest 64 %). However, as a confirmatory test WB is highly specific when compared to the IFA or ELISA as the method allows the visualisation of the virus antigen(s) recognised by serum samples. Compared to IFA and ELISA this technique is time-consuming and costly and the high specificity seen with WB may be accompanied by a decrease in sensitivity (Waltrip *et al.*, 1995).

1.5.2.3. Enzyme-linked immunosorbent assay (ELISA).

ELISA assays allow the detection of viral antigen or antibody using a solid-phase assay system and also provided sensitive and specific diagnostic tests of acute and chronic infection (Bode *et al.,* 2001). This technique provides a non-reader-dependent quantitative result as opposed to IFA and Western blotting (WB). The BDV triplet ELISA uses a cocktail of anti-BDV monoclonal antibodies and a polyclonal antiserum for antigen capture and detection. This triple ELISA detects BDV antigens, antibodies and circulating immune complexes (CIC) in test serum (Horimoto *et al;* 1997; Bode *et al.,* 2001; Yamaguchi *et al.,* 1999) by binding them to BDV monoclonal antibodies or monoclonal antibody antigen mixtures "captured" by BDV which have previously been bound to the ELISA plate (figure 1.10). Conjugated secondary antibodies are added and an enzymatic reaction will give a coloured product. This colour is proportional to the amount of antigen, antibody or circulating immune complexes in the test sample.

ELISA is regarded as a very sensitive method for the detection of BDV (Bode *et al.,* 2001) but there are specificity concerns with these ELISAs as for example some sera that are seronegative by WB can give a positive result in ELISA due to non-specific antibody (Evengard *et al.,* 1999). This may be bypassed with the use of a "blocking" step using soluble BDV antigens to confirm the specificity of the antibodies binding to the BDV antigens on the ELISA plate in order to improve the specificity of the test (Yamaguchi *et al.,* 1999).

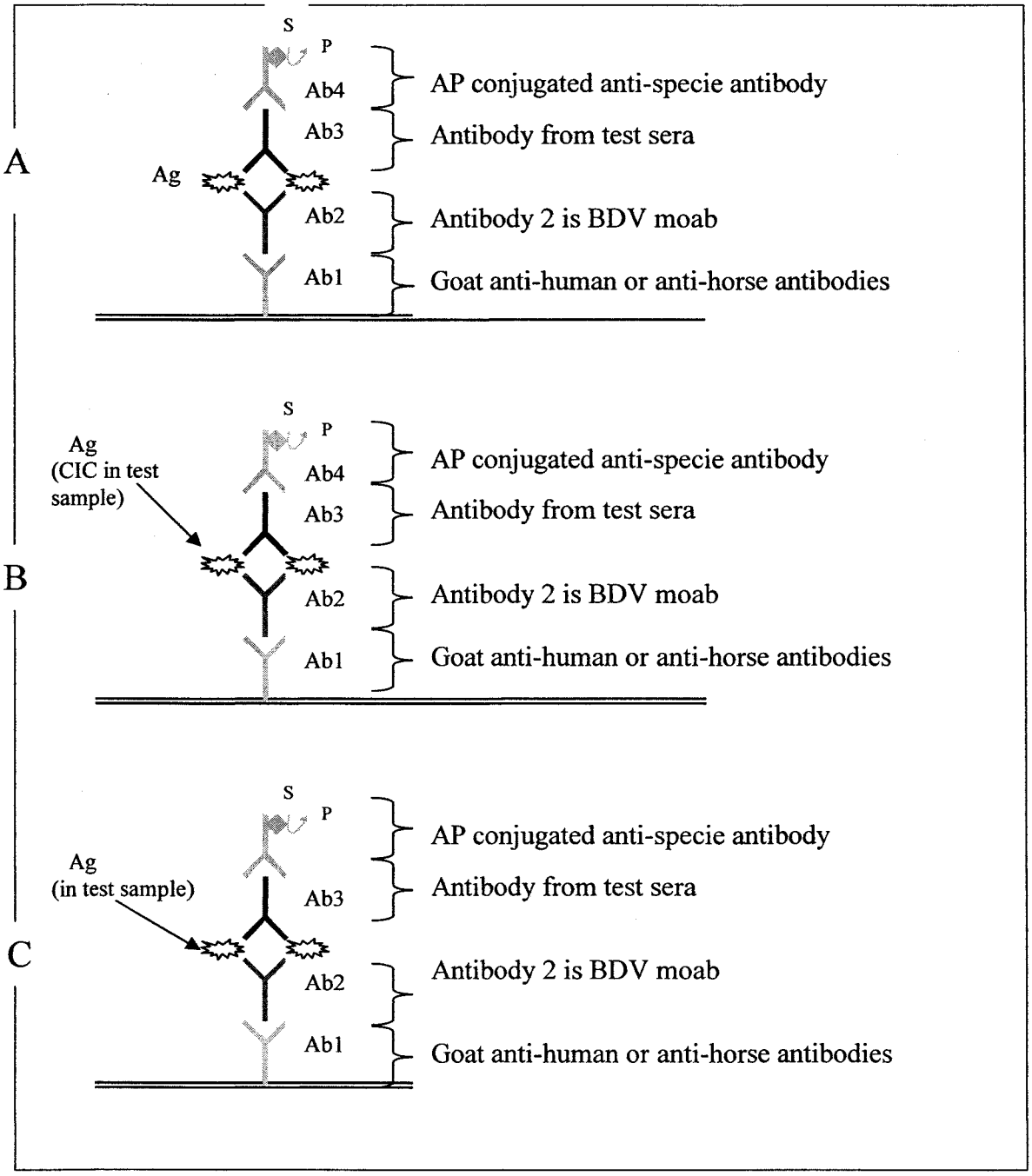

Figure 1.10: BDV diagnosis by triplet ELISA test. A: the antibody assay, the antigen is prepared from ultrasonicated BDV infected OL cells; B: the CIC assay, a direct detection of immune complexes from serum; C: the antigen assay, a double antibody detection of antigen from serum. The enzyme-linked antibody (AP conjugated anti-specie antibody or Ab4) is bound to chromogenic substrate (S) and gives a coloured product (P) which will enable measurements of positive samples.

On the ELISA triplet essay, the three BDV markers are assayed on the same solid phase and compared to single ELISAs the seroprevalence is very high, up to 62 % in the human population (Bode *et al.*, 2001).

1.5.3. Molecular diagnosis for BDV nucleic acid detection

The development of molecular techniques has made it possible to detect BDV RNA in cells, tissues and the blood of BDV infected animals and humans (Sierra-Honigman *et al.*, 1995; Nakamura *et al.*, 1996; Bahamani *et al.*, 1996; Kishi *at al.*, 1995a). These methods include Southern and Northern blot analysis, *in situ* hybridization, polymerase chain reaction (PCR) and gene arrays (Fugiwara *et al.*, 1997; Billich *et al.*, 2002; Pyper *et al.*, 1998; Nakamura *et al.*, 2000).

Southern blot analysis was developed in the 1970s and involves the fractionation of DNA with restriction enzymes, followed by transfer of DNA to a solid membrane support and detection of virus-specific sequences using radiolabelled viral nucleic acids (Fujiwara *et al.*, 1997).

Southern blot and hybridization assays have been used by several investigators for determination of BDV cDNA and has been found to be useful when used for a

relatively small number of samples and for confirmation purposes following nested RT-PCR as it has the additional benefit of uncovering low levels of PCR products not visible by ethidium bromide staining (Zimmermann *et al.,* 1994; Sauder & de la Torre, 1998).

In situ hybridization is a good histological technique that uses a radioactively or enzymatically labelled complementary strand of BDV sequence-specific nucleic acid probe that binds to BDV RNA in infected cells (Carbone *et al.,* 1991). However, for epidemiological studies its use could be disadvantageous due to the relatively lengthy process and subjective results due to the low concentrations of BDV RNA, BDV RNA degradation and non-BDV-specific probe binding (Sierra-Honigham *et al.,* 1993; Fugiwara *et al.,* 1997; Nakamura *et al.,* 2000).

Northern blot analysis on the other hand is an analogous method for the detection of RNA sequences where molecular weight markers are included in the blot to confirm the location of known sizes of BDV proteins and technical performance. It is a commonly used technique in many BDV studies and has been used to screen both animals and psychiatric patients with variable rates of detection (Fukuda *et al.,* 2001; Chen *et al.,* 1999a; Carbone, 2001).

Polymerase chain reaction (PCR), possible the most sensitive and widely used method for detection of viral nucleic acids is the gold standard for BDV nucleic acid detection. RT-PCR for BDV was initially developed to identify very small amounts

of BDV RNA in animals but was soon used to detect BDV nucleic acids in cells, fluids and tissue of both animals and humans (Sierra-Honigmann *et al.*, 1993; Iwata *et al.*, 1998; Amsterdam *et al.*, 1985; Bode *et al.*, 1993). In this technique RNA must be isolated and converted into cDNA using a reverse transcriptase enzyme and is referred to as reverse transcription (RT)-PCR, prior to amplification (figure 1.11).

The use of a thermostable DNA polymerase, specific primers and multiple cycles of amplification, which comprises melting of the double-stranded DNA template, annealing of the oligonucleotide primers to the template and extension of the primers by the thermostable DNA polymerase, permits the creation of new DNA chains and the consequent amplification of fragments of viral nucleic acid from serum, cell or tissue samples. Nucleic acid obtained from these reactions can be sequenced for confirmation purposes (Rott & Bencht, 1995; Rott & Bencht, 2001; Schwarz *et al.*, 2001).

First strand synthesis

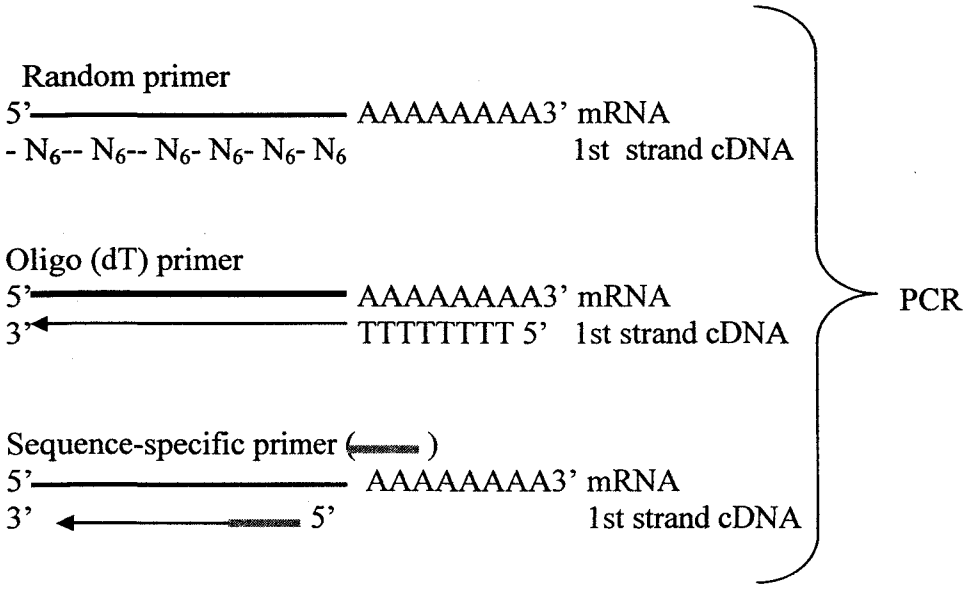

Figure 1.11: Schematic diagram of RT-PCR from Promega's PCR Protocols & Reference (Promega, 2006)

Direct identification of viral nucleic acids in body fluids or cells offers several important advantages over serological/immunological methods for assessment of BDV infection and determining the frequency of non-symptomatic persistent infections with BDV (Saunder & de la Torre, 1998). The major feature of this assay is that it is more sensitive than the immunology-based assays, by several orders of magnitude, resulting in accurate detection of very low levels of viral nucleic acid (Sauder & de la Torre, 1998).

Although RT-PCR is generally believed to be highly sensitive, the presence of RT-PCR inhibitors, such as heparin or haemoglobin in a blood sample or other contamination artefacts, may result in false-negative or false-positive results respectively. Moreover there have been reports on samples that were seropositive and subsequently tested negative by RT-PCR suggesting an irregularity in BDV shedding and/or variability in the genome and thus BDV infection may be missed. The use of RT-PCR methods for the detection of BDV nucleic acids in clinical and laboratory specimens has been controversial and as a consequence the use of a range of techniques to confirm results is considered good practice (Sauder & de la Torre, 1998; Ritch *et al.*, 1993; Kishi *et al.*, 1995a).

Several investigators have reached different conclusions on the molecular detection of BDV but whether these were influenced by confounding factors such as primers, proteins, low levels or absence of BDV or general technique is hard to tell. For instance in relation to humans Iwata *et al.* (1998) searched for p24 RNA using BDV p24 specific primers (Kishi *et al.*, 1995b) in peripheral blood mononuclear cells (PBMCs) of psychiatric patients and blood donors by nested reverse transcriptase PCR (RT-PCR) and found prevalence of 4 % in psychiatric patients and 2 % in blood donors. However, Kishi *et al.* (1995b) who initially used the same primers found higher prevalence in psychiatric patients (37 %). Rather than looking for only p24 Sauder *et al.* (1996) increased their chances of detection of BDV by searching for both BDV p24 and p40 and their results were astonishing in that 63 % of psychiatric

patients were positive. The sequences they obtained (from human PBMCs) also exhibited both a high degree of inter- and intrapatient conservation and a close genetic relationship to animal-derived BDV sequences (Saunder *et al.*, 1996), which contrasted to subsequent reports where sequence analysis of the human BDV p24 cDNA showed high nucleotide sequence conservation of p24 but a distinct nucleotide mutation in comparison with horse BDV sequences (Iwata *et al.*, 1998; Kishi *et al.*, 1995b).

In general RT-PCR has proved to be an extremely useful technique for the detection of BDV RNA in biological samples for both humans and animals and has always detected a higher prevalence of BDV in psychiatric patients as compared to healthy donors (Iwata *et al.*, 1998; Kishi *et al.*, 1995a; Bode *et al.*, 1995; Ygata-Yi *et al.*, 1996; Sauder *et al.*, 1996).

1.6. Aims and hypotheses

In 2000, the Public Health Laboratory Service, Health and Safety Executive; the Ministry of Agriculture, Fisheries and Food; the Welsh Development Agency; and the Wales Innovation Relay Centre organised a workshop on Borna Disease Virus, to review information on the diagnosis, pathology, and epidemiology of BDV in humans and animals (Thomas *et al.*, 2000). The author attended the seminar in which over 6 dozen delegates from 9 countries including, the veterinary and public health policy makers in developing surveillance and research programs, took part. The meeting brought up a number of questions:

1. Is BDV present in UK animal populations?
2. Is there clinical disease compatible with Borna disease in animals in the United Kingdom?
3. Are human populations in the United Kingdom likely to be exposed, and if so is there any evidence that exposure could lead to clinical illness?

This study was then initiated to answer the questions on BDV prevalence and to determine whether Borna Disease Virus (BDV) causes a persistent infection in humans and animals in the UK and to determine if there was any link between BDV infection and psychiatric disease.

The prevalence of BDV was measured in 95 bipolar patients in different stages of depression, with each subject undergoing a psychiatric evaluation and mood assessment. A number of healthy individuals (matching age/sex) from both psychiatric hospital and from non hospital origins were also studied to make up the controls. A large group of horses were also studied to determine the seroprevalence of BDV in UK equine.

The hypotheses for this study were:

- Borna Disease Virus (BDV) causes a persistent infection in human and animals and antibodies, antigens and circulating immune complexes (CIC) may be detected in infected individuals.
- Markers of BDV infection as well as virus can also be detected by serological and molecular methods in healthy individuals although at a much lower prevalence than in those individuals that are chronically depressed.

Detailed objectives were as follow:

1. To determine the seroprevalence of BDV infection in horses by triplet ELISA
2. To determine the seroprevalence of BDV infection in horses by IFA
3. To determine the prevalence of BDV infection in horses by RT-PCR
4. To determine the seroprevalence of BDV infection in human by triplet ELISA

5. To determine the seroprevalence of BDV infection in human by IFA
6. To determine the prevalence of BDV infection in human by RT-PCR
7. To determine the seroprevalence of BDV infection in horses by triplet ELISA
8. To confirm seroprevalence testing by IB
9. To confirm BDV in cells by isolation

CHAPTER 2:

2. MATERIALS AND METHODS

2.1. Brief introduction

Borna Disease Virus (BDV), the causative agent of Borna Disease (BD) is responsible for meningo-encephalomyelitis in animals and possibly psychiatric disorders in humans. BDV does not restrict itself to one particular area of the brain or the body and can be detected by a broad range of diagnostic methods. However, BDV has a relatively low replication rate and the highest titres, about 10^6 tissue culture infection dose ($TCID_{50}$ ml^{-1}) are found in the brain (Herzog *et al.*, 1984; Herzog, & Rott, 1980 and Morales *et al.*, 1988).

The major serological methods currently used for screening of BDV infection in animals and humans are the highly sensitive and specific anti-BDV p40 and p24 ELISA (Yamaguchi *et al.*, 1999). These serological techniques may also be used for the routine screening of blood products and thus may prevent the transmission of BDV by this route. The detection rate of BDV markers for both animals and man by this method appears to be considerable (up to 50 %) although at this point it is impossible to confirm whether these individuals are actually infected with BDV.

In this study BDV antibody, antigen and circulating immune complexes were detected by triplet ELISA. This was followed by IFA and RT-PCR which were used as confirmatory techniques.

The isolation of BDV from a patient and all of the relevant techniques are at the end of the section were used to confirm the diagnosis of human BDV infection.

In other studies BDV has been isolated from autopsied human brain tissue and animal brain and blood (de la Torre *et al.*, 1996b) whilst in this study BDV was isolated from human blood which would make it easier to detect and isolate the virus from live patients although it was noted that BDV is more likely to be concentrated in other parts of the body.

2.2. Test samples

In the majority of cases 10 ml of blood was collected, from both humans and animals, into EDTA. However, serum samples were also collected occasionally.

2.2.1. Human samples

Ethical approval for the Research Project was obtained from the joint ethics committee of the Newcastle and North Tyneside Health Authority, University of Newcastle upon Tyne and University of Northumbria at Newcastle and written consent was obtained from patients and hospital controls prior to donation of blood.

2.2.1.1. Patients:

Mood disorder group (Group 1)

Blood was collected from 95 patients attending the Psychiatric Outpatient Care, Leazes Wing (East), ward 31 - Royal Victoria Infirmary (RVI), Newcastle-upon-Tyne between 2000 and 2001. All patients were diagnosed according to the Diagnostic & Statistical Manual of the Psychiatric Association – revised fourth edition criteria (DSM-IV) as patients with major depressive disorders or bipolar disorders (American Psychiatric Association, 1994). According to this same criteria these patients were sub-classified according to their specific subgroups with 35 patients being diagnosed with major depressive disorder (MDD), 19 with bipolar I rapid cycling (BPIRC), 30 with Bipolar I not-rapid cycling (BPInotRC), 6 with Bipolar II rapid cycling (BPIIRC), and 5 with Bipolar II not-rapid cycling (BPIInotRC) conditions. One patient presented with symptoms that did not fulfil the guidelines for DMS-IV, but presented with the signs of BDV infection which included disturbance in learning and memory, ataxia, visual impairment and social withdrawal (Bode *et al.,* 1993). Clinical examination did not show apparent signs of viral infection but all patients had been treated with oral dexamethasone 16 hours prior to blood collection.

Sex, occupation and other details of patients involved in this study were recorded and/or shown in appendix II-a. There were 48 female and 45 males in this group. Details of gender and sex are shown in table 2.1.

2.2.1.2. Controls:

Hospital control (Group 2)

The hospital control group was made of 32 healthy subjects in direct contact with mood disorder patients at the Psychiatric Outpatient Care, Leazes Wing (East), ward 31 - Royal Victoria Infirmary (RVI), Newcastle-upon-Tyne. They did not undergo a full psychiatric evaluation and did not undergo a clinical or physical evaluation but all responded to a questionnaire in order to fit in the "healthy" criteria before blood donation. This questionnaire included questions on their age, sex, occupation, clinical health status, recent and past infections, present and recent medication, physical and emotional status. Details of each volunteer's health and activities were discussed in a confidential setting prior to blood collection. The inclusion selection criteria included people in an age range of 18 to 65 years, working full time in ward 31, and those people considered "healthy" without any form or sign of infection. For this study purpose and in general "healthy" means that the person felt well and could perform normal activities. However, for some people with chronic condition such as

diabetes or high blood pressure, "healthy", in this case, also meant those individuals being treated with the condition under control, thus allowing these people to donate blood. Individuals on other types of medication (e.g. antibiotics, hepatitis B immunoglobulin and insulin), those with high temperature, with cold or flu, those who had been exposed to hepatitis, people with or recently treated for sexually transmitted disease and people who had been outside the hospital setting for at least 30 days prior to blood collection were excluded. The age and sex of members of group 2 individuals is shown in table 2.1. Full details for each member including their occupation are found in appendix II-b.

Blood donors (Group 3)

401 blood donor samples from the Newcastle upon Tyne Transfusion Centre were also included in this study as a second healthy control group. A psychiatric evaluation was not made on any of these individuals, but the donor's health history was thoroughly checked, at the author's request, by professionals at the blood transfusion centre who were fully aware of the inclusion and exclusion criteria for this study, as described above for group 2. Only blood that fitted the established criteria was provided. The healthy status of each donor was previously discussed as part of the donation process before any blood was collected. Moreover, each donor had received a brief examination during which temperature, pulse, blood pressure

and blood count (haemoglobin or hematocrit) were measured as part of a routine for all blood donors. Also, all resultant blood taken from healthy subjects was screened for certain infection conditions (e.g. HIV, Hepatitis B).

Although the blood centre has full details of each donor, the author was unable to report them here, apart from age and sex (in appendix II-c), due to the bureaucracy involved in obtaining a formal consent from each donor.

Group	**Total**	**Sex**		**Mean age**	
	both sexes	Male	Female	Female	Male
1. Patients	95	48	45	48	46
2. Hospital controls	32	15	17	48	46
3. Blood donors	401	188	213	45	43

Table 2.1: The three human groups: Patient group, hospital control group and blood donor group, by age and sex. The patient group includes all in the 5 sub-classification of mood disorders (MDD, BPInotRC, BPIInotRC, BPIRC, BPIIRC); the sex of the individuals in the three groups matched in terms of percentages. Similarly, the mean age for both male and females also matched.

2.2.2. Horse Samples

Horses were selected for the present study, above other animals, based on the evidence that horses were the first natural host for BDV identified (Ludwig *et al.*, 1988), and that BDV infection and exposure in horses has been reported in several countries including the UK (Thomas *et al.*, 2005). Moreover, it has also been reported that the neurologic manifestations of BDV infection in horses resembles the neuropsychiatric disorders in humans (Bode & Ludwig, 1997) which makes them the first animal of choice for BDV detection.

For this study, several veterinary clinics in the North east region were contacted and provided with a briefing. Three clinics made themselves available, but only one (in Stocksfield, Hexham), was able to follow the selection criteria established by the author. This included selection of horses, drawing of 10 ml of blood samples from apparently healthy horses into heparinised tubes and providing the samples with details of horses involved in the study within an hour of blood collection.
A second batch of samples representing the British Isles were acquired from Dr Malcolm Banks (Veterinary Laboratories Agency, Weybridge).

No attempt to recruit samples from horses showing clinical signs of Borna disease was made as for the animals there was no intention of comparing data from diseased and non-diseased animals. In this study as in this case the aim was to determine if horses in UK had been exposed or infected with BDV.

In summary, samples were collected from a total of 272 horses and were divided into the two following groups:

2.2.2.1. Regional equine group (Horse group 1):

This group consisted of 78 blood samples and were provided by a Veterinary clinic in the Northern region of England (Hexham, Northumberland). Details of name and age of horse were supplied with 14 exceptions (appendix III-a).

2.2.2.2. National equine group (horse group 2):

This group consisted of 196 equine serum samples, which had been collected during a routine national surveillance scheme and were kindly provided by the Veterinary Laboratories Agency. These were partially characterized by age (24 out of the 196) and geographical location (appendix III-b).

2.3. Serological Methods for BDV detection

Serological methods are the main method for BDV detection and diagnosis of infection. The major serological methods currently used for screening for BDV infection in animals and humans are ELISA and IFA which are highly sensitive and specific tools. In this study anti-BDV p40 and p24 antibodies were measured (Bode *et al.*, 2001; Yamaguchi *et al.*, 1999). A brief explanation of the ELISA and IFA are shown in sub-section 1.5.2.

2.3.1. Enzyme immuno assays (ELISA)

The triplet tests for the detection of antigens, antibodies and circulating immuno-complexes were developed in the Robert Koch Institute (Bode *et al.*, 2001) and allow the detection of BDV antigens p40 and p24 present in blood plasma/serum (pAg; p40/p24 heteromers included), antibodies to BDV proteins and CIC. Three different assays with the same solid support were used. The software analyses of each sample on each of the three assays were based on the full complement analysis of the blanks, positive and negative controls. A cut-off point of 0.1, an arbitrary number, was a reference point to determine negative and positive optical density (OD) values read at 405 nm. All blanks values were to have an OD of <0.1. The negative control was a sample pre-determined negative after three consecutive analysis with an OD of <0.1.

On average the negative control value after subtracting the blank was 0.04. The positive control was a sample also pre-determined positive after three consecutive analyses with an OD of ≥0.1. An example of the positive control calculation is found in sub-section 3.2. All samples with values in the range of 0.079 and 0.149 were retested.

A list of reagents is found in appendix I.

Materials

Monoclonal antibodies (Moabs)

Monoclonal antibodies against BDV antigen were a kind gift from Dr Liv Bode, Robert Koch Institute, Germany, and they were:

- Anti-BDV p40 and p24 mouse monoclonal antibodies (W1HO and Kfu2, hybridoma supernatants IF- Titre 1:2000) produced from persistently infected asymptomatic Balb/c mice
- A polyclonal rabbit anti-BDV antiserum was also donated from the same source

Borna Disease Virus

≻ Ultra-sonicated BDV strain V infected oligodendroglial (OL) cells (titre 10 focus forming unit ml^{-1})

Secondary antibodies and conjugates

All secondary antibodies and conjugates were supplied by Jackson Immuno research Labs, West Grove, Pa, USA and included:

≻ Alkaline Phosphatase (AP)-conjugated affinipure goat anti-rabbit IgG, FcFragment-specific (absorbed against human serum proteins)

≻ AP-conjugated Affinipure goat anti-mouse IgG, FcFragment-specific (absorbed against human, bovine, equine serum proteins)

≻ AP-conjugated affinipure goat anti-human IgG, Fc Fragment-specific (absorbed against mouse, bovine, equine serum proteins)

≻ AP-conjugated affinipure goat anti-horse IgG, Fc Fragment-specific (absorbed against human serum proteins)

2.3.1.1. ELISA technique: Antigen Assay

Maxisorp Immuno Modules (Nunc, Roskilde, Denmark) were coated with 100 µl of 1.8 µg ml^{-1} goat anti-mouse IgG, Fc Fragment diluted in 10 mM sodium phosphate pH 7.6 containing 250 mM sodium chloride, overnight at 4°C. The plates were then washed three times in 0.9 % sodium chloride containing 0.05 % Tween 20 (Ultra wash plus, Dynatatech Labs, Chantilly, VA, USA). Equal volumes of anti-BDV p40 and p24 monoclonal antibodies (Kfu2 and W1HO fluid) diluted 1:500 in PBS containing 0.05 % Tween 20 (PBS-T) were mixed and 100 µl was added to each well. Plates were incubated for 1 hour at 37°C and then washed three times as previously described. 200 µl of serum diluted 1:2 in PBS-T was double diluted to 1/16 down the plate in PBS-T and the plates were incubated for 2 hours at 37°C. After washing 100 µl of polyclonal rabbit anti-BDV serum diluted 1:1000 in PBS-T was added to each well and the plates were incubated for a further 2 hours at 37°C. The plates were washed again and 100 µl AP-conjugated Goat Anti-Rabbit IgG (FcFragment-specific) diluted 1:3000 in Tris buffered saline containing 0.05 % Tween 20 (TBS-T) was added to each well and the plates were incubated for 2 hours at 37°C. Finally the plates were washed and 100 µl of freshly prepared substrate *p*-nitrophenylphosphate (pNPP- SIGMA) in 1M Diethanolamine buffer (pH 9.8) + 0.5 mM magnesium chloride was added to each well and incubated for 5 minutes at room temperature. Figure 2.1 shows the bind step for each antibody, anti BDV antibody and antigen.

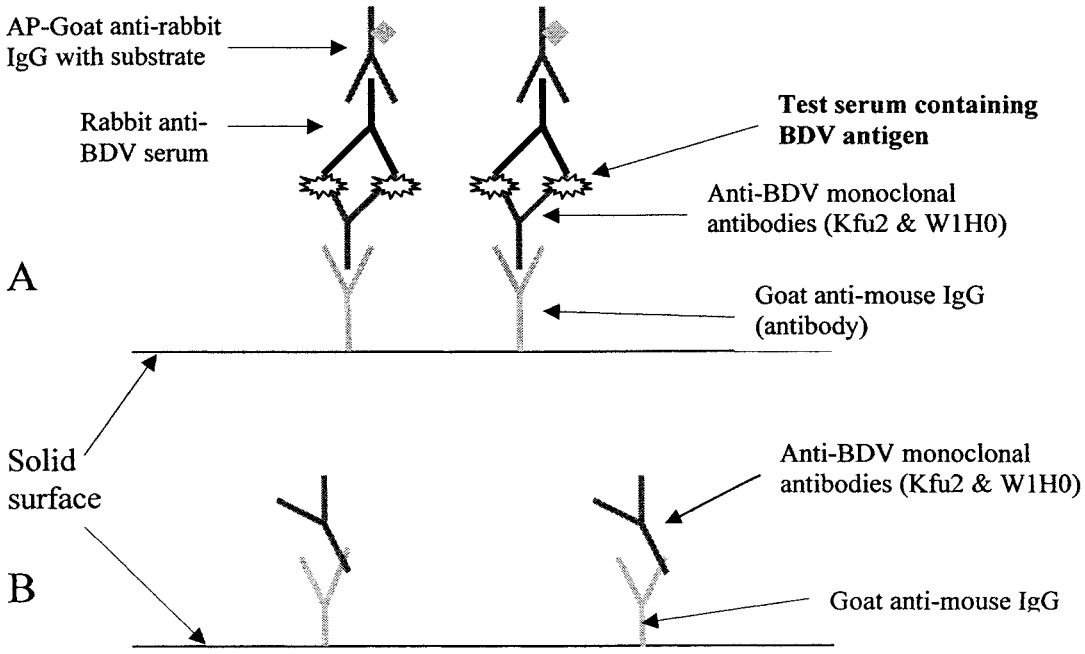

Figure 2.1: BDV ELISA test designed to measure BDV antigen present in serum.

A: Positive test - Plates were coated with goat anti-mouse IgG and incubated to ensure binding the antibody to the surface of microplate wells (solid surface). Anti-BDV monoclonals and then test sample were diluted and added to the plate. To detect the presence of antigen-antibody binding, polyclonal rabbit anti-BDV serum and alkaline phosphatase conjugated goat anti-rabbit IgG serum were added. Enzyme activity was assayed and the amount of coloured product is proportional to the protein present in the solution. B: Negative test – A negative control (or negative sample) was assayed in parallel. In this case no antigen can bind to the moab as there will be none in the sample. Thus no colour develops.

The enzymatic reaction was stopped by the addition of 50 µl of 3 M sodium hydroxide, and the optical density read at 405 nm in Microplate Reader (Dynatech Microplate Reader MRX, USA). All samples were run in duplicate. Positive and negative controls were run along with the unknown samples each time to validate the results. A positive result for BDV antigen was determined by relating the optical density value (OD) to the specific cut-off point of >0.1. OD values of less than 0.1 were scored as negative. All samples with values in the range of 0.079 and 0.149 were retested.

2.3.1.2. ELISA technique: The Antibody Assay

Maxisorp Immuno Modules (Nunc Roskilde, Denmark) were coated with 100 µl of 1.8 µg ml^{-1} AffiniPure Goat Anti-Mouse IgG (FcFragment-specific) in 10 mM sodium phosphate pH 7.6 containing 250 mM sodium chloride and incubated overnight at 4°C. The plates were washed three times in washing buffer (0.9 % sodium chloride + 0.05 % Tween 20). 100 µl of BDV p40 and p24 mouse monoclonal antibodies (moabs) (W1H8 and Kfu2), diluted 1:500 in PBS-T was added to each well and plates were incubated for 1 hour at 37°C and then washed three times as previously described. Antigen was prepared by ultra-sonication of BDV strain V infected oligodendroglial (OL) cells (titre 10 focus forming unit ml^{-1}). 100 µl of this preparation diluted 1:300 in PBS-T was added to each well and incubated overnight at 4°C. After washing 200 µl of serum diluted 1/100 in PBS was

added to all wells and double diluted down the plate (to 1/16) in PBS-T then incubated for 2 hours at 37°C. After washing, 100 µl of alkaline phosphatase (AP)-conjugated AffiniPure Goat Anti-Human IgG, Fc Fragment-specific, diluted 1:3000 in TBS-T was added to each well and the plates were incubated for 1 hour at 37°C (for horse samples, AP-conjugated AffiniPure Goat Anti-Horse IgG, FcFragment-specific, at the same dilution measured). The plates were washed and 100 µl of freshly prepared substrate *p*-nitrophenylphosphate (pNPP) in 1 M Diethanolamine buffer + 0.5 mM magnesium chloride was added to each well and incubated for 5 minutes at room temperature. A representative diagram showing the principle of this assay is shown in figure 2.2.

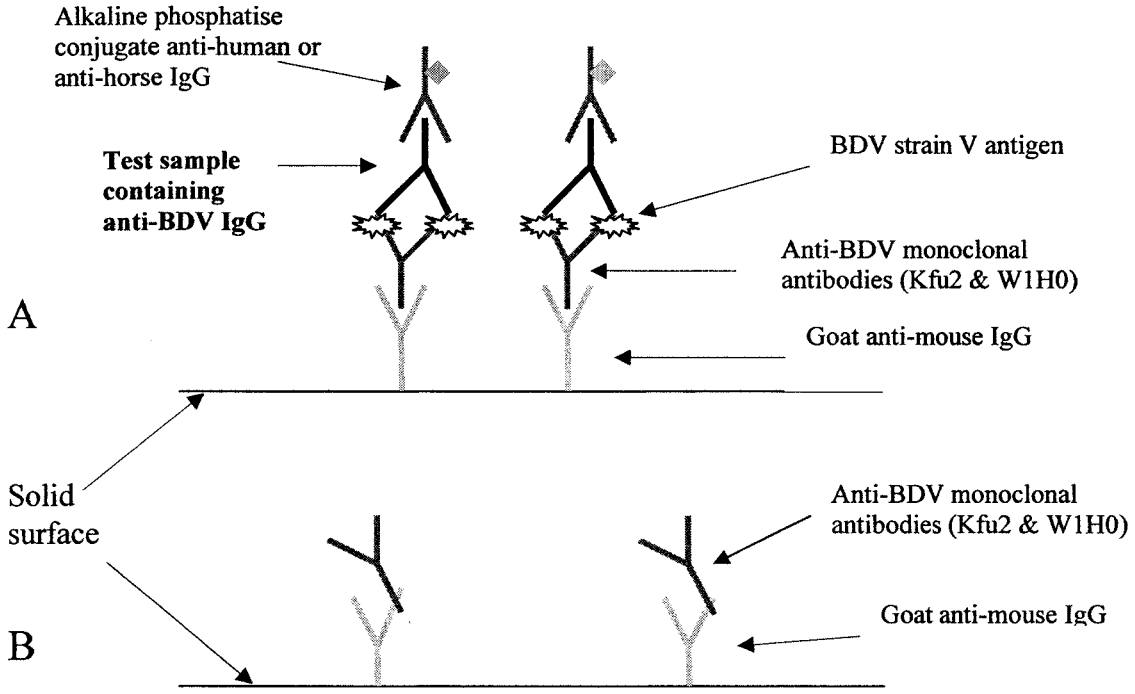

Figure 2.2: BDV ELISA test designed to measure anti-BDV IgG present in serum. A: Positive test - Plates were coated with goat anti-mouse IgG and incubated to ensure binding of the antibody to the surface of microplate wells (solid surface). Anti-BDV monoclonals, ultrasonicated BDV antigen and test serum were diluted and added to the plate in sequence. AP conjugated anti-human or anti-horse IgG was then added and finally enzyme activity was assayed. The amount of coloured product was proportional to the antibody present in the solution. B: Negative test – Negative control (or negative sample) were assayed in parallel.

The enzymatic reaction was stopped by the addition of 50 µl of 3 M sodium hydroxide, and read at 405 nm in Dynatech Microplate Reader MRX. Positive and

negative values were determined according to the established conditions as described previously (antigen assay).

2.3.1.3. ELISA technique: The CIC assay

Maxisorp Immuno Modules (Nunc, Roskilde, Denmark) were coated with 100 µl of 1.8 µg ml^{-1} goat anti-mouse IgG, Fc Fragment diluted in 10 mM sodium phosphate pH 7.6 containing 250 mM sodium chloride, overnight at 4°C. The plates were then washed three times in 0.9 % sodium chloride containing 0.05 % Tween 20 (Ultra wash plus, Dynatatech Labs, Chantilly, VA, USA). Equal volumes of anti-BDV p40 and p24 monoclonal antibodies (Kfu2 and W1HO) diluted 1:500 in PBS containing 0.05 % Tween 20(PBS-T) were mixed and 100 µl was added to each well. Plates were incubated for 1 hour at 37°C and then washed three times as previously described. 200 µl serum diluted 1/20 in PBS was double diluted down (to 1/16) the plate in PBS-T and the plates were further incubated for 2 hours at 37°C. After washing, 100 µl of alkaline Phosphatase (AP)-conjugated AffiniPure Goat Anti-Human IgG, Fc Fragment-specific diluted 1:3000 in 20 mM TBS-T was added to each well and incubate for 1 hour at 37°C (for horse samples, AP-conjugated AffiniPure Goat Anti-Horse IgG, FcFragment-specific at the same dilution was used). Finally the plates were washed and 100 µl of freshly prepared substrate *p*-nitrophenylphosphate (pNPP- SIGMA) in 1 M Diethanolamine buffer (pH 9.8) + 0.5

mM magnesium chloride was added to each well and incubated for 5min at room temperature.

A diagram representative of the method is shown in figure 2.3.

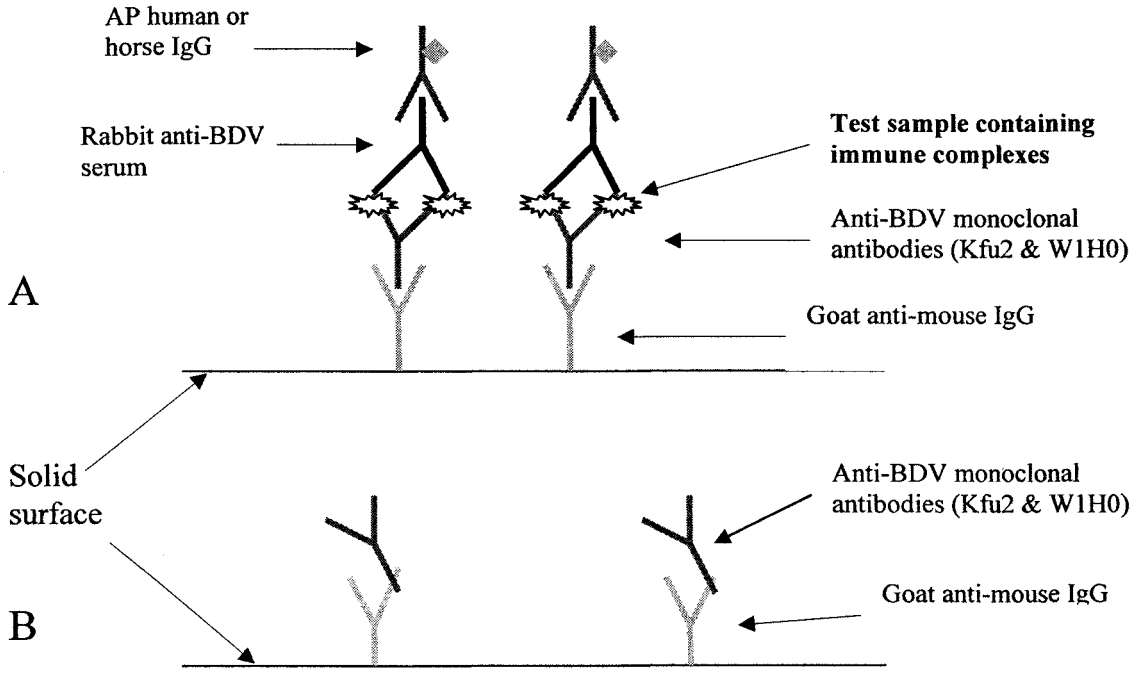

Figure 2.3: BDV ELISA test designed to measure BDV specific immune complexes present in serum. A: Positive test - Plates were coated with goat anti-mouse IgG and incubated to ensure binding of the antibody to the surface of microplate wells (solid surface). BDV monoclonals and the test serum were diluted and added to the plate in sequence. Any antigen present in the test sample will bind to the anti-BDV monoclonal antibodies. Immune complexes were labelled by the addition of AP conjugated goat anti-human or goat anti-horse IgG. The enzyme activity was assayed and the amount of coloured product was proportional to the complexes present in the solution. B: Negative test – Negative control (or negative sample) was assayed in parallel.

2.3.2. Immunofluorescence assay

The BDV Immunofluorescence assay was used to detect anti-BDV antibodies in test samples and to determine the cellular location of BDV particles by fluorescence microscopy.

The fluorescent dyes fluorescein and rhodamine were conjugated to anti-p40 and p24 monoclonal antibodies. The procedure for this assay involves two phases. Firstly primary YRB cells line were infected with BDV and seeded onto cover slips as described in sub-section 2.3.2.1. Secondly the assay to measure specific anti-BDV antibody in test samples was carried out as described in sub-section 2.3.2.2. A diagrammatic representation of this method is shown in figure 2.4.

Materials

Virus

- Ratpool (BDV strain V)

Moabs

BDV anti p40 and p24 mouse moabs (W1, Kfu2, hybridoma supernatants IF- Titre 1:2000), Kindly supplied by Robert Koch Institute.

Secondary antibody conjugates

- Rhodamine-CyTm3 conjugated affinipure goat anti-mouse IgG, F (ab')^2 fragment specific (TRITC); code 115-1655-072; antibody concentration = 1.5 mg/ml; working dilution 1:1000
- Fluorescein-conjugated affinipure goat anti-horse IgG, F (ab')^2 fragment-specific (FITC); code 108-096-008; antibody concentration = 1.2 mg/ml; working dilution 1:100
- Fluorescein-CyTM2 conjugated affinipure goat anti-human IgG, F (H + L); code 109-225-088; antibody concentration = 1.2 mg/ml; working dilution 1:100

Cells

- *Primary cell line:* Young Rabbit Brain (YRB) culture. A culture of Young Rabbit Brain cells, from a 2 day old rabbit, was set-up at the Sheffield Medical School. Cells

were infected with BDV in the Microbiology Laboratory at Newcastle General Hospital, Newcastle upon Tyne.

➢ *Established cell line:* Infected and uninfected Human Oligodendroglial (OL) cells and Young Rabbit Spleen cells (YRS) (a kind gift of Free University of Berlin) were also maintained in DMEM containing 5 % FCS.

2.3.2.1. Culture of primary cell line for the IFA technique

Prior to the IFA technique, cover-slips were prepared with BDV infected rat brain cells as follows. The brain of a new-born rabbit was removed from the cerebral cavity and placed into a sterile petri dish containing 70 % ethanol for a few seconds. The brain mass was washed three times with sterile PBS and finally with DMEM containing 5 % FCS. The brain was disrupted through a large gauge needle (no 1) until a suspension was generated. 10 ml of medium (DMEM containing 5 % FCS) was added to a petri dish and 0.5 ml of brain suspension was added and mixed with a pipette. Plates were incubated in a CO_2 incubator at 37°C and the medium changed after 2 days.

Cells were viewed every day and 4 days later the medium was changed. Between 7 and 10 days cells were ready for the first passage. At the 3rd passage cells were used for culture onto cover slips and infection with BDV. (Details of cell passage are found in sub-section 2.5.1). To this end sterile 19 mm diameter cover slips were

placed into wells of a 24 well plate. Once the primary YRB lines were passaged three times 2.5 x 10^4 YRB cells were re-suspended into 25 ml of medium and 1 ml of BDV (ratpool 50 ffu pre determined by IFA) was added to the cells. 1 ml of medium/cell/virus mix was added to each cover slip and the plates were incubated at 37°C in 5 % CO_2 for 5 days. The cover slips were then fixed in acetone for 10 minutes and then stored at -20°C for up to 1 year.

2.3.2.2. Immunofluorescence assay technique

Serum samples were heat inactivated for 30 minutes at 56°C prior to testing. Cover-slips coated with cells were thawed and washed in PBS containing 1 % Newborn Calf Serum (NCS) for 5 seconds and then air dried.

Test serum at fold dilution of 1/10, 1/20, 1/40 and 1/80 was added to BDV-p40/p20-specific monoclonal antibody (W1H0/KFU2) diluted 1/100 in PBS. 20 µl of this diluted solution was dropped on to a glass plate and covered with the coverslip, which was previously fixed with cells. These coverslips, containing cells sunken in the solution of serum/monoclonals, were incubated in a damp chamber for 30 minutes at 37°C and then washed twice for 10 minutes in PBS containing 1 % FCS. A mixture of goat anti-human or horse IgG-FITC (1/100) and anti-mouse IgG-

TRITC (1/1000) conjugates (Jackson Immuno Research Labs) was made in PBS. 200 µl of this conjugate mix was placed on to a glass plate and then was once again covered with the cells on coverslips that were previously incubated and washed. Coverslips were further incubated for 30 minutes in damp chamber at 37°C and finally washed twice for 10 minutes in PBS containing 1 % FCS. Finally, 10 µl of glycerol diluted 1:1 in PBS was added to a slide and covered with the coverslip. Cells were viewed under the easy-change filter fluorescent microscope (OLYMPUS BX 40). Cells were scored as either positive or negative for both virus (as measured by TRITC-red fluorescence) and anti-viral antibody (as measured by FITC- green fluorescence). Analysis of fluorescence was determined in the presence of the positive and negative controls. Positive controls were cells infected with polyclonal anti-BDV serum produced in experimentally infected animals (rat-pool) and negative controls were uninfected YRB cells. These controls, as well as the anti-BDV monoclonal antibodies demonstrated that the immunofluorescence signal reflected localization of the target BDV protein in the cell. Further controls used during testing was the omission of all antibodies to determine if there was any auto-fluorescence of the tissue, and competition testing (pre-incubation of primary antibody for 30 min with the BDV antigen) to check for non-specific binding of the primary antibody. Only cells with 3 or more foci were counted as positive as it is very unlikely to have non-specific binding in a cell with more than 2 foci.

2.4. Detection of BDV RNA in cells of peripheral blood cells and/or plasma/serum by RT-PCR

Diagnosis of BDV *in vitro* is currently performed by the demonstration of either virus-specific antibodies, antigen, and/or circulating immune complexes and this is crucial for BDV diagnosis but molecular detection of RNA in serum, PBMCs or cerebrospinal fluid gives further insight into the prevalence and molecular pathology of BDV. This extra insight is provided by sensitive real-time and RT-PCR techniques and sequencing of the BDV amplicons, which are leading to tracing different BDV strains, analyzing the geographic distribution of BDV and revealing intra species (and possibly interspecies) transmission routes and sources of BDV by phylogenetic trees based on sequences of single BDV genes (Bode & Ludwig, 2003; Herzog & Rott, 1980; Bode *et al.,* 2001; Iwata *et al.,* 1998; Kao *et al.,* 1993).

In order to verify whether BDV nucleic acid was present in human and equine plasma/sera and peripheral blood cells, and confirm the results obtained by serological techniques, a reverse transcriptase reaction followed by nested-PCR amplification was carried out on the samples. The reaction assays were based on that developed by Bode (1995) and the primers used in these reactions were also designed by Bode (Ludwig & Bode 2000) and Schwarz (Schwarz *et al.,* 2001). These authors amplified the region within the first open reading frame (ORF-I) encoding the p40 nucleoprotein, which has been reported to be relatively conserved within the BDV

genome (Walker *et al.*, 2000). Amplified products were analysed by gel electrophoresis using a 100 bp DNA ladder under appropriate conditions.

Materials

Samples

As per sub-section 2.2.

Cells

Infected and uninfected OL and YRB were used at protein concentration of 1.5 μg/μl

Primers

Two sets of primers were used for each reaction (Bode's method; Ludwig & Bode 2000) in order to amplify a 441 bp fragment of the BDV ORF I (p40 nucleoprotein) (38A, 38B, 186, 606). The Qiagen method (Schwarz *et al.*, 2001) required the primers p40 s and p40 as in order to amplify a 449 bp fragment of the BDV ORF I (single reaction method). The primer name, sequence and position are found in table 2.2.

Primer	Sequence	Nucleotide position in BDV genome	Polarity
38A	5'-GTCACGGCGCGATATGTTTC-3'	242-261	Sense
38B	5'-CTTCTTACTCCAGTAAAACGC-3'	989-969	Anti-sense
186	5'-GCCTTGTGTTTCTATGTTTG-3'	277-296	Sense
606	5'-ATTCTTTACCTGGGGACTCA-3'	717-698	Anti-sense
p40s	5'-ACGCCCAGCCTTGTGTTTCT-3'	270-720	Sense
p40as	5'-AATTCTTTACCTGGGGACTCAA-3'	270-720	Anti-sense

Table 2.2: Partial BDV sequences used in the RT-nested-PCR and nucleotide positions in relation to primers

Each primer was made to 20 μM sub-stock by dilution in 1 x TE and then stored at -20°C.

2.4.1. Blood cell separation

Histopaque (Sigma diagnostics) was bought to room temperature, protected from light and 3.0 ml pipetted into the chamber of an accuspin tube. The tubes were centrifuged at 800 g for 30 seconds at room temperature. 4 ml of fresh anti-

coagulated whole blood was added to the tubes which were then centrifuged at 800 g for 15 minutes at 20°C. The plasma fraction was collected and stored at -70 °C. Mononuclear cells were collected and transferred into a clean centrifuge tube and washed with 10 ml of PBS at 250 g for 10 minutes at room temperature. This washing step was repeated twice and the final pellet was re-suspended in 1 ml cell culture medium.

2.4.2. Cell quantification

Cells were counted with a haemocytometer. An equal volume of 0.1 % Trypan blue was added to the cells to determine the proportion of viable cells in the suspension by dye exclusion.

2.4.3. *RNA isolation*

RNA was isolated from cells, plasma or serum for subsequent use in polymerase chain reaction. Three different RNA extraction methods were used according to the starting material for maximal yield. Thus for RNA extraction from PBM cells, serum and/or plasma the Trizol reagent was used. For RNA extraction from OL and YRB

cells the RNase mini kit (Qiagen) was used whilst for RNA extraction from whole blood the QIAamp RNA Blood Mini Kit was used.

2.4.3.1. Extraction of RNA from PBMCs, plasma and/or serum with Trizol reagent

PBMCs and plasma were separated from whole blood by Histopaque centrifugation as described (sub-section 2.4.1.). 1 ml of Trizol reagent was added to either 5-10 x 10^6 of cells or 100 µl of plasma and mixed by repetitive pipetting. The homogenised samples were then incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. 200 µl of chloroform was added to each sample and the tubes were shaken for 15 seconds and then further incubated at room temperature for 2 to 3 minutes. Samples were centrifuged for 15 minutes at 11 000 *g* at 4 °C, following which the mixture separated into a lower red, phenol-chloroform phase, an interface, and a colourless upper aqueous phase. As RNA remains exclusively in the aqueous phase, the red-coloured organic phase was discarded. For RNA precipitation the aqueous phase was transferred to a fresh tube and the RNA was precipitated by the addition of 500 µl isopropanol. After incubation at room temperature for 10 minutes and centrifugation at 11 000 g for 10 minutes at 2 - 8 °C the RNA formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed and the RNA pellet washed once with 1ml of 75 % (v/v) ethanol. After mixing the samples were centrifuged at 7 000 g for 5 minutes at 2-8 °C and the

supernatant was discarded. The RNA pellet was briefly air-dried for 5-10 minutes under a warm lamp and then 20 µl of RNAse free water was added to re-dissolve the RNA samples. The samples were incubated for 10 minutes at 55 °C and then the samples were stored at either –20°C or –70°C until further use. 10 µl of the RNA (of each) was diluted 1/10 in RNA free water for the determination of RNA concentration (sub-section 2.4.4.).

2.4.3.2. RNA extraction from whole blood by QIAamp RNA Blood Mini Kit

The QIAamp RNA Blood Mini Kit was used for the isolation of cellular RNA from 5ml of whole human and horse blood in EDTA. The procedure for RNA isolation by this method was similar to the next although this method had the advantage over the others in that cells did not have to be separated from whole blood before RNA isolation. Following the manufacturer's instructions, this procedure completely removed RNases, contaminants, and enzyme inhibitors yielding high-quality RNA. Blood cells were selectively lysed and white cells collected by centrifugation. White cells were then lysed using highly denaturing conditions, which immediately inactivated RNases. After homogenisation using the QIAshredder spin column, the samples were applied to the QIAamp spin columns. Total RNA bound to the QIAamp membrane and contaminants were washed away, leaving pure RNA to be eluted in 30–100 µl RNase-free water (provided with the kit) for direct use in RT-PCR.

2.4.3.3. Isolation of total RNA from YRS and Oligo Cells using the RNAse mini Kit

YRS cells and Oligo cells grown in a monolayer (10^5 cells) in cell-culture flasks and/or in cell culture dishes (sub-section 2.5.1.) were trypsinised and collected as a cell pellet prior to lysis.

For disruption of the cells the pellet was loosened from the tube (by flicking) and 600 µl of RLT Buffer (for ~1 X 10^5 cells) was added. Finally tubes were vortexed and mixed by pipetting.

Similarly to the RNA extraction method from whole blood, the lysate was then directly pipetted onto the QIAshredder spin column which was placed in a 2 ml collection tube and this was then centrifuged for 2 minutes at 8000 g. 600 µl of 70 % ethanol was added to the homogenised lysate and mixed well by pipetting (not centrifuged). 600 µl of the sample, including any precipitate that was formed was added to a RNeasy mini column and placed in a 2 ml collection tube for centrifugation. The tubes were centrifuged for 15 seconds at 8 000 *g* after which the flow-through was discarded. The RNeasy column was replaced into the collection tube and 700 µl of RW1 buffer was added to the columns and centrifuged for 15 seconds at 8 000 *g* to wash the column. The flow-through was again discarded and the RNeasy column was transferred to a new 2 ml collection tube. 500 µl of RPE

Buffer was pipetted onto the RNeasy column and then centrifuged for 15 seconds at 8 000 *g* to wash the column. The flow-through was discarded and a final 500 µl of RPE Buffer was added to the RNeasy column, and centrifuged for 2 minutes at 8 000 *g* to dry the RNeasy silica-gel membrane.

The RNeasy column were placed into a new 2ml collection tube and centrifuged at 8 000 *g* for 1 minute to eliminate any chance of buffer carryover. The RNeasy mini column was carefully removed from the collection tube and transferred to a new 1.5 ml collection tube for elution. 50 µl of RNase-free water was added directly onto the RNeasy silica-gel membrane, and centrifuged for 1 minute at 8 000 *g* for elution of RNA. This step was repeated twice. Finally, 10 µl of the eluted product was placed into a clean micro-centrifuge tube for RNA measurements and the remainder of the material was stored at – 20°C.

2.4.4. RNA Quantification

Two quartz cuvettes were washed with RNase-free water. One of them was used as a blank containing 1ml of RNase-free water and the other cuvette the RNA sample diluted 1/100 in Milli-Q Water.

The concentration of RNA in each preparation was determined by measuring its absorbance at 260 nm (A260) and 280 nm (A280). RNA purity was indicated by the fact that RNA does not absorb at values lower than 240 nm whilst chemicals such as phenol and chloroform do at 260 nm thus allowing the determination of contamination.

2.4.5. *RT-PCR*

Two methods were used to amplify regions of BDV first open reading frame (BDV p40). These methods would then provide data on the sensitivity of a simple RT-PCR and a nested RT-PCR.

2.4.5.1. RT-nested-PCR

The protocol for One-Step-nested RT-PCR was adapted from that originally described by Bode and Stoyloff (1995). Approximately 5 x 10^3 BDV copies per reaction were used to carry out the reaction with the QIAGEN OneStep RT-PCR Kit and the protocol was optimised for 2 μg of total RNA. Additional concentrations of 10 μg, 10ng, 1 ng and 1 pg BDV RNA were also used to measure the sensitivity. Reverse transcription and PCR were carried out sequentially in the same tube of the

PCR. All components required for both reactions were added during the set-up, and there was no need for additional components once the reaction had been started.

First round PCR

Template RNA, primer solutions, dNTP Mix, 5x QIAGEN OneStep RT-PCR Buffer, and RNase-free water were thawed and placed on ice. The master mix was prepared according to table 2.3.

Reagents	**Volume per Reaction**	**Final Concentration**
RNA free H2O	Variable	-
5 x QIAGEN OneStep RT-PCR Buffer (contains 12.5 mM MgCl2)	10.0 µl	1x
DNTP-Mix (QIAGEN) (containing 10 mM of each dNTP)	2.0 µl	400 µM
Primer 38A (20 µM) (external BDV-p40 primer with anti-genomic polarity)	1.5 µl	0.6 µM
P38B (20 µM) (external BDV-p40 primer with genomic polarity)	1.5 µl	0.6 µM
QIAGEN OneStep Enzyme Mix (Omniscript Reverse Transcriptase, sensiscript Reverse Transcriptase, HotStarTaq DNA Polymerase)	2.0 µl	-
RNAse inhibitor* (Rnasin 40 U/µl) (Promega)	0.5 µl	20 U/reaction
Template RNA** (added at step 4)	Variable	1 pg - 2 µg/reaction
Total volume	50.0 µl	-

Table 2.3: Mix for 1st round PCR and relative concentration of reagents per reaction (Bode method). *Reagent not supplied in the kit. Purchased from Promega UK Ltd. ** Template extracted either from the plasma, serum or cells.

The master mix contained all the components required for RT-PCR except the template RNA, which was added at the end with a volume 10 % greater than that required for the total number of reactions performed. A negative control (without template RNA) was included in all experiments. The master mix was dispensed into PCR tubes, and template RNA (2 μg/reaction) was added to the individual PCR tubes.

The thermal cycler program was set according to the program outlined in table B4. The RT-PCR program was started whilst the PCR tubes were still on ice and the tubes only placed into the thermal cycler when it had reached 50°C.

Nested PCR (HotStarTaq DNA Polymerase)

RT-nested-PCR was carried out using a sample acquired from the first round as the template. Total RNA was afterwards extracted from both biological samples. Extraction of total RNA from cells was achieved with Trizol reagent and RT- PCR was afterwards performed. The nested PCR mix reaction of set up according to table 2.4.

The procedure for this reaction was similar to the first round, which required the thawing and mixing of reagents to prepare the master mix with a 10 % greater

volume than the one required for the total number of PCR assays to be performed. Again the use of a negative control (without template DNA) and positive controls was essential.

Reagents	**Volume per Reaction**	**Final concentration**
RNAse free H2O	37.25 µl	
10 x QIAGEN PCR Buffer	5.0 µl	1x 1.5 mM MgCL2
DNTP-Mix (MBI Fermentas)	5.0 µl	200 µM of each dNTP
Primer 186 (20 µM) (external BDV-p40 primer with anti-genomic polarity)	1.0 µl	0.4 µM
Primer 606 (20 µM) (external BDV-p40 primer with genomic polarity)	1.0 µl	0.4 µM
HotStarTaq DNA Polymerase (5 U/µl (QIAGEN)	0.25 µl	1.25 U/reaction
Template cDNA (first round)	0.5 µl	
Total volume (µl)	50.0 µl	

Table 2.4: Mix for nested-PCR for a single sample mix and relative concentration of reagents per reaction (Bode method). Template was the product of the first round PCR.

With the second round reactions, it was not necessary to keep PCR tubes on ice as non-specific DNA synthesis could not occur at room temperature due to the inactive state of HotStarTaq DNA Polymerase.

The template DNA (~1 µg/reaction) was obtained from aliquots from the reverse transcriptase reaction.

Finally the thermal cycler was set up as shown in table 2.5.

Reaction	**Temp.(°C) Round 1**	**Time (Min) Round 1**	**Temp. (°C) Round 2**	**Time (Min) Round 2**
1.Reverse transcription	50	30	-	-
2.Revertase inactivation/ Polymerase activation	95	15	95	3
3.Denaturation	94	1	94	1
4.Annealing	56	1	56	1
5.Extension	72	1	72	1.5
6.Final extension	72	10	72	10
7.Storage	4		4	
Step 3,4,5 in 33 loops				

Table 2.5: Amplification conditions for RT-nested-PCR (1st and 2nd round; Bode method).

The table describes thermal cycler programs and the program includes steps for both reverse transcription and PCR. The PCR amplification segment started with an initial heating step of 95°C for 15 minutes for HotStarTaq DNA Polymerase activation.

2.4.5.2. Qiagen RT-PCR (Schwarz method)

RNA isolation from cells and plasma/serum was carried as previously described. Similar to the first reaction of Bode's method, approximately 5 x 10^3 BDV copies per reaction was used to carry out the reaction with the QIAGEN OneStep RT-PCR Kit (table 2.6). Additional concentrations of 10 µg, 10 ng, 1 ng and 1 pg were also used to compare the sensitivity of the methods.

Reagents	Volume per Reaction	Final Concentration
RNA free H2O	12.5 µl	-
5 x QIAGEN OneStep RT-PCR Buffer (contains 12.5 mM MgCl2)	10.0 µl	1x
DNTP-Mix (QIAGEN) (containing 10 mM of each dNTP)	2.0 µl	400 µM
Primer p40as (20 µM) (external BDV-p40 primer with anti-genomic polarity)	1.5 µl	100 pM
Primer p40a (20 µM) (external BDV-p40 primer with genomic polarity)	1.5 µl	100 pM
RNAse inhibitor(Rnasin 40 U/µl) (Promega)*	0.5 µl	20 U/reaction
Template RNA**	20 µl	1 pg - 2 µg/reaction
QIAGEN OneStep Enzyme Mix***	2.0 µl	-
Total volume	50.0 µl	-

Table 2.6: Reagent mix for RT-PCR and relative concentration of reagents per reaction (Schwarz method). *Reagent not supplied in the kit. Purchased from the Promega UK Ltd, Delta House, Chilworth Research Centre Southampton, SO16 7NS. ** Template extracted either from the plasma, serum or cells. *** Omniscript Reverse Transcriptase, sensiscript Reverse Transcriptase, HotStarTaq DNA Polymerase.

The program of the thermal cycler was set up according to table 2.7.

Reaction	**Temp.(°C)**	**Time**
1.Reverse transcription	50	30 minutes
2.Revertase inactivation/Polymerase activation	95	15 minutes
3.Denaturation	94	30 seconds
4.Annealing	56.5	45 seconds
5.Extension	72	1 minutes
6.Final extension	72	10 minutes
7.Storage	4	
Step 3,4,5 in 45 loops		

Table 2.7: Amplification conditions for the RT- PCR (Schwarz method).

2.4.6. Electrophoresis of the PCR products using the 100 bp DNA Ladder

50 ml of agarose gel (1.5 %) in TAE buffer was prepared to which 2.5 µl of ethidium bromide was added. The gel was placed into an electrophoresis tank (Pharmacia GNA – 100) and TAE buffer was poured over the gel until it was completely submerged.

Samples were prepared in fresh micro-centrifuge tubes by mixing 2 µl of blue/orange 6 x loading dye (G188A) to 10 µl of PCR products. These mixtures were loaded into the wells of the submerged gel. Normally the first well was loaded with the control molecular weight ladder solution made up of 2 µl of the loading dye to 10 µl of Promegas's 100 bp DNA ladder.

The electrophoresis was run under 100 volt and 200 mA for 1 hour. The results were viewed under UV light (BIO-RAD Gel Doc 2000).

2.5. Isolation of BDV from a psychiatric patient

In this part of study 2 major techniques were used for virus isolation:

1. Cell-culture which is a fundamental technique for the growth and propagation of the virus.

2. Virus isolation and quantification.

Foci assays were used for the quantification of virus. Further tests were used for the detection of BDV specific antigen, antibodies and RNA in the sub-cultured cells using enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay (IFA), Western blotting (WB) and RT-PCR to confirm the presence and identity of newly isolated virus.

All virological manipulations were carried out in the level 3 containment laboratories at either the microbiology laboratory, Newcastle General Hospital, Newcastle Medical School or the Microbiology and Virology Departments at the Free University of Berlin by the author.

Materials

Samples

10 ml of blood was collected from three individuals as shown in table 2.7. These individuals were previously involved in the prevalence study (details in section 3.5) and the results presented are these of the triplet-ELISA test. Further informed consent was obtained from each individual.

Cells

The permanent human oligodendroglial cell line (OL) was used for isolation of human BDV. The cells were obtained from Prof. Hans Ludwig (Germany) (originally acquired from Dr Y Iwasaki, from the Wistar Institute, Philadelphia, PA, USA in the early eighties), propagated over 100 times and treated for mycoplasma contamination (Bode *et al.,* 1996). These OL cells were grown in DMEM and used at an initial cell concentration of 1 x 10^4 cells/ml. A permanent BDV infected cell line generated from human cells was also cultivated in tissue culture flasks. This line was infected with a rat adapted virus and carried the designation of TL.

2.5.1. Subculture of attachment-dependent cell lines growing in cell culture (the established cell line passage)

The established human oligodendoglial cell line (OL) was used for propagation and isolation of human BDV. Following aspiration of spent media 10-15 ml of PBS without Ca^{2+} /Mg^{2+} was added to each flask to rinse the cells. After aspiration, 1ml of trypsin/EDTA solution was added to each flask. The flasks were rotated to cover the monolayer with trypsin and then incubated for 2-10 minutes at 37°C with inspection at 5 minute intervals to ensure that all the cells were detached and floating. When

cells were detached, 5 ml of completed growth medium was added to the flasks and mixed to disperse the cells and neutralise the action of trypsin.

Before subdivision into new flasks, 15 ml of complete culture medium (Dulbecco's modified Eagle's minimum essential medium (DMEM) sterile filtered + 5 % (v/v) Newborn calf serum (NCS) + 0.1 % Penicillin-Streptomycin) was added to each of the required number of new 75 cm^2 cell culture flasks, which were labelled with date, cell ID and passage level (ratio 1:4). Infected and non- infected cells were maintained in a humidified incubator under 5 % CO_2 at 37°C until cells were confluent (about 3 days). Sub-culturing of the cells was then repeated.

2.5.1.1. Cell storage

Cells were frozen in liquid nitrogen for long term preservation. Adherent cells were release from the culture vessel with trypsin as described previously (section 2.5.1.). Detached cells were re-suspended in 1 ml of culture medium without antibiotics to neutralise the action of trypsin. 75 µl of this mixture was dispensed into a 2 ml sterile cryovial and 35 µl of freezing solution containing 25 % FCS and 20 % DMSO was added to the vial and mixed well. The vial was placed in an ice bath for 5 minutes and a further 40 µl freezing medium was added to the cells. Vials were kept at -70°C for 2 hours, wrapped in cotton wool and then placed into liquid nitrogen for long term storage.

2.5.1.2. Retrieval of cells from liquid nitrogen

Vials were removed from liquid nitrogen and after checking that the caps were secure they were placed into warm water with gentle agitation for about 2 minutes. The cryotubes were dried with absorbent paper and rinsed with 70 % ethanol. The cell suspension from each cryotube was transferred to a 50 ml sterile tube and 1.0 ml of culture medium was added drop wise with gently agitation to the cell suspension and then a further 24 ml of culture medium was added slowly to the cells. Cells were pelleted by centrifugation at 800 *g* for 5 minutes and the supernatant was discarded. The cell pellet was re-suspended in 15 ml of fresh medium and the cells were transferred to a 75 cm^2 culture flask and cultured as previously described.

2.5.2. Infection of OL cells with BDV from patients

5 ml of OL cells at 2.5 x 10^4/ml were plated into a 6 well plate (Corning Science Products) and incubated at 37°C for 3 hours. 200 µl of freshly isolated patient PBMCs (2 x 10^5 cell/ml), ultrasonicated in a Brason Sonifier for 20 cycles, was added to the OL cells. 200 µl of plasma was added to a further well and the same amount of whole blood to another. Two of the remaining wells were not infected and

therefore only contained OL cells (neg. controls) and one further well was infected by the addition of 200 µl of the BDV positive homogenate ratpool (1x 10^5ffu). This procedure was carried out in duplicate and the plates were incubated for 6 days at 37°C in a humidified atmosphere containing 5 % CO_2. Cells were harvested every 3 to 4 days for serological and molecular analysis (sub-section 2.3 and 2.4).

2.5.3. Confirmation of BDV infectivity by means of focus assay

Suspensions

Virus suspension:

2.5 ml of DMEM containing 100 µl of the virus

Sample suspension:

50 µl of OL, OL/TL, PBMC or plasma in 450 µl DMEM supplemented with 0.1 % Pen/Strep and 10 % FCS, adjusted to contain 4 x 10^5 cells per ml).

Uninfected cell suspension:

OL cells or YRS at 10^4 cells l^{-1} (10 ml DMEM to 1 plate of trypsinised cells)

2.5.3.1. Virus Titration Test (Focus assay) technique

The virus titre was established for quantification of BDV in the infected cells and is given as focus forming units, as calculated by an Enzyme-linked Immuno-Monolayer Assay (EIMA). 100 µl of OL or YRS cells at 10^4 cells ml^{-1} was added to each well of a 96 microtitre plate (NUNC plastic-ware, UK). The plates were incubated for 3 hours at 37°C under 5 % CO_2. 15 µl of sample suspensions from the original infected samples were added to the plates and diluted logarithmically down the plate and further incubated for further 4 days.

2.5.3.2. Cell ELISA (EIMA)

Cells were prepared as described by the titration method and incubated for 96 hours at 37°C and then screened by EIMA for the presence of virus.

The supernatant was discarded and the cells were fixed with 50 µl of formalin at room temperature for 30 minutes. The formalin was discarded and 50 ml of PBS

containing 1 % TritonX-100 was added for 30 seconds to permeate the cells. The PBS-TritonX was removed and the cells washed with PBS containing 1 % newborn calf serum (PBS-NCS). The cells were then incubated with 50 ml of monoclonal antibody KFU2 diluted 1 in 100 in PBS, for 1 hour at 37°C. The cells were then washed with PBS-NCS. 50 µl of peroxide-conjugated anti-mouse IgG (Fc) diluted 1 in 500 in PBS-NCS was added to the cells and plates were incubated for 1 hour at 37°C. After a further wash in PBS-NCS the cells were incubated with 50 µl of substrate/stain mix for 30 minutes at room temperature and in the dark. The reaction was stopped with tap water. Virus positivity was determined subjectively by viewing the cells under the microscope, with positive cells staining a red/purple colour. The number of virus containing cells was calculated by multiplying the number of positive cells by the dilution factor and further 5 to determine the ffu/ml.

2.5.4. Confirmation of BDV infectivity by ELISA, IFA, WB and RT-nested-PCR

2.5.4.1. BDV infectivity confirmation by ELISA, IFA and RT-nested-PCR

At every passage cells were tested for the presence of BDV markers by ELISA, IFA and RT-nested-PCR as per sub-section 2.3 and 2.4.

2.5.4.2. BDV infectivity confirmation by Western Blotting (WB)

WB was used to identify BDV p40 and p24 proteins in the samples and simultaneously determine its molecular weight. The procedure involved the size separation of the proteins in the mixture by polyacrylamide gel elecrophoresis (PAGE), the addition of anti BDV antibody, the transfer of the separated proteins to a membrane while retaining their relative position and the detection of BDV proteins by its specific reaction with the test plasma or control sera and determination of its size relative to standard proteins.

Western blotting was performed essentially as described by Bode *et al,* (2001). Preparation and running of SDS - Polyacrylamide Gel Electrophoresis was conducted using the Laemmli method and a mini-gel system. Briefly a 10 % polyacrylamide separating gel was poured with a 5 % stacking gel. Serum samples were diluted 1/10 in water and 15 µl of diluted serum and 10 µl reducing Laemmli-loading buffer was heated at 100 °C for 5 minutes. 25 µl of this sample mix was loaded into a plate and electrophoresis was carried out for about 1 hour. To determine the molecular weights of the samples, a standard marker was run simultaneously (BioRad). The positive control was made of a sample of ratpool run on the same gel to establish monoclonal antibody binding. After electrophoresis the separating gel was put into a blotting sandwich. The gel was subsequently placed

into the tank, filled with transfer buffer and transfer of proteins to the nitrocellulose was carried out at 100 V at room temperature. The nitrocellulose membrane was then incubated for 1 hour at 37°C in blocking buffer. The nitrocellulose was washed twice with PBS-T and BDV proteins present in test sera and in monoclonal antibody were detected in the membrane by horse-radish peroxidase-conjugate goat anti-human IgG (Sigma) diluted 1:1000 in TBS, which was incubated for 2 hours at room temperature and visualised with enhanced chemoluminescence detection technology (Pierce, Rockford, ILL).

CHAPTER 3:

3. RESULTS

3.1. General summary of samples tested

The number of samples analysed are given below with full details of results in appendix II and III. A general summary of individual groups can also be found in sub-section 2.2.

3.1.1. Human samples

Group 1- 95 patients (mood disorder group) from Newcastle Psychiatric Hospital sub-grouped according to DSM-4 criteria.

Group 2 - 32 Healthy subjects from Newcastle Psychiatric Hospital

Group 3 - 401 blood donors – Group 3

3.1.2. Equine samples

Group 1 - 78 were collected from a local veterinary surgeon in Newcastle

Group 2 - 196 were collected during a National surveillance programme

In total 274 horse samples were analysed by all assays. They were not classified as diseased horses or controls as a clinical evaluation was not made at the origin point, thus results are presented as a whole for these samples.

3.2. Detection of BDV markers in human samples by serological analysis

BDV markers detected by serological analysis were mainly the soluble s-antigen, which consists of three proteins (60kDa, 38/40kDa and 25kDa) (Ludwig *et al.*, 1988). These antigens provoke the major immune response in BDV infection and antibodies against the nuclear antigen are predominant. Tests were run independently and blinded.

The triplet ELISA was carried out after the optimum dilution of sera was established. This was determined using a range of infected and uninfected controls, double diluting them down an ELISA plate, at 5 different times under the same conditions and the values found for the calibration curve were used to calculate the lower detection limit. Optimal serum dilutions were: 1:2 for detecting BDV antigen, 1:20 for detecting CICs, and 1:100 for detecting serum antibodies. The detection limit for each of the BDV-specific components was determined to be an OD of 0.1 at 405 nm.

All blanks were to have an OD of <0.02. Negative control was a sample pre-determined negative after three consecutive analysis with an OD of <0.1. In average the negative control value after subtracting the blank was of 0.04. Positive control was a sample also pre-determined positive after three consecutive analyses with an

OD of ≥0.1. Positive control had a mean value of 1.13. 95 % confidence intervals for the mean were calculated and all samples found between 0.079 and 0.149 were retested.

In the case of Immunofluorescence, the results were established on a range of four doubling sera dilutions. The optimum dilution for testing the plasma antigen was found to be 1:20 and cells were classified as positive if two or more nuclear fluorescent foci were present in a single cell and observed with both fluorescein and rhodamine.

3.2.1. Detection of BDV markers in human samples by triplet ELISA

3.2.1.1. Detection of BDV antigen (pAg)

BDV antigens were detected by means of a direct ELISA. Briefly, antigen in the test serum binds to a monoclonal antibody previously coated onto the plate. Remaining BDV epitopes were detected with a heterologous hyper-immune rabbit serum and a corresponding enzyme conjugated anti-rabbit immunoglobulin anti-sera. The enzyme activity of the bound material was determined by the addition of the substrate and the green-yellow colour formed is proportional to the amount of

antigen in the test sample. The results for the three groups tested are shown in figure 3.1.

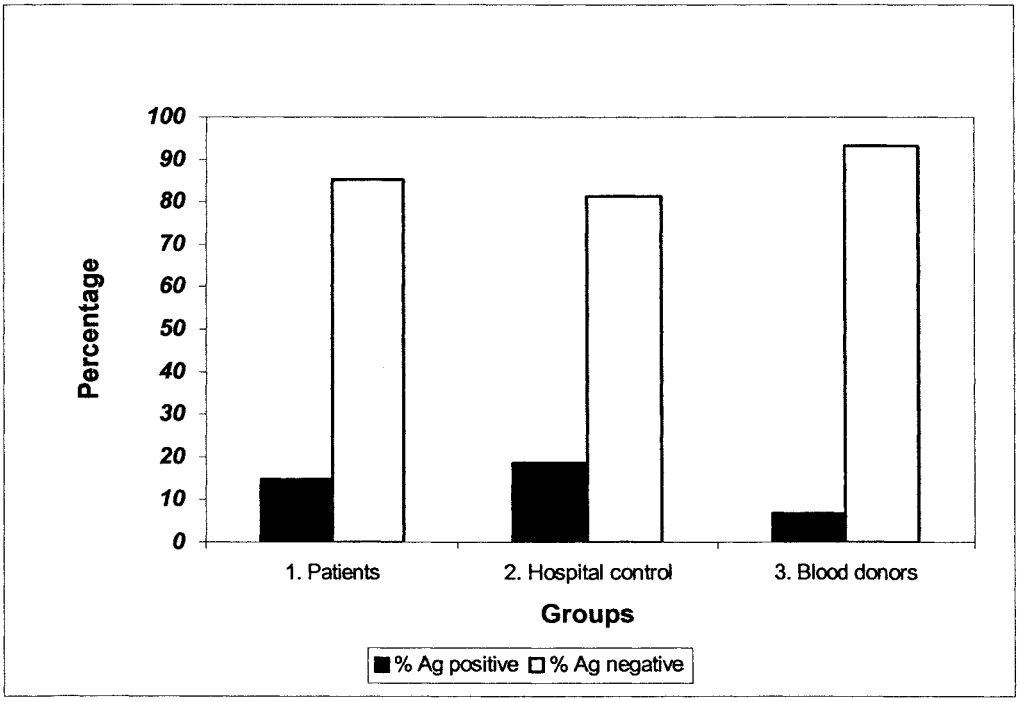

Figure 3.1: Results of antigen detection by the ELISA test. The percentage of individuals testing positive/negative for BDV for each of the three groups is shown. Ag = antigen.

In general more than 80 % of serum samples tested ware negative for BDV antigen by the triplet ELISA. About 15 percent of mood disorder patients (group1) were positive for antigen and ~18 % of psychiatric hospital controls (Group2) were also positive. Both of these groups had significantly higher levels of BDV antigen positivity as compared to the blood donor pool (group 3) ($\chi^2 = 10.14$ p=0.006). The mean OD of the positive samples in each of these groups is shown in figure 3.3.

3.2.1.2. Detection of BDV circulating immune complexes (CIC)

Circulating immuno complexes (CIC) were detected using the anti-p40 and anti-p24 monoclonal antibodies as capture antibody. Stable complexes consisting of N and P monomers or dimmers and most probably N-P heteromers were detected by the addition of anti-human IgG and human specific conjugate. The results for the three groups are shown in figure 3.2.

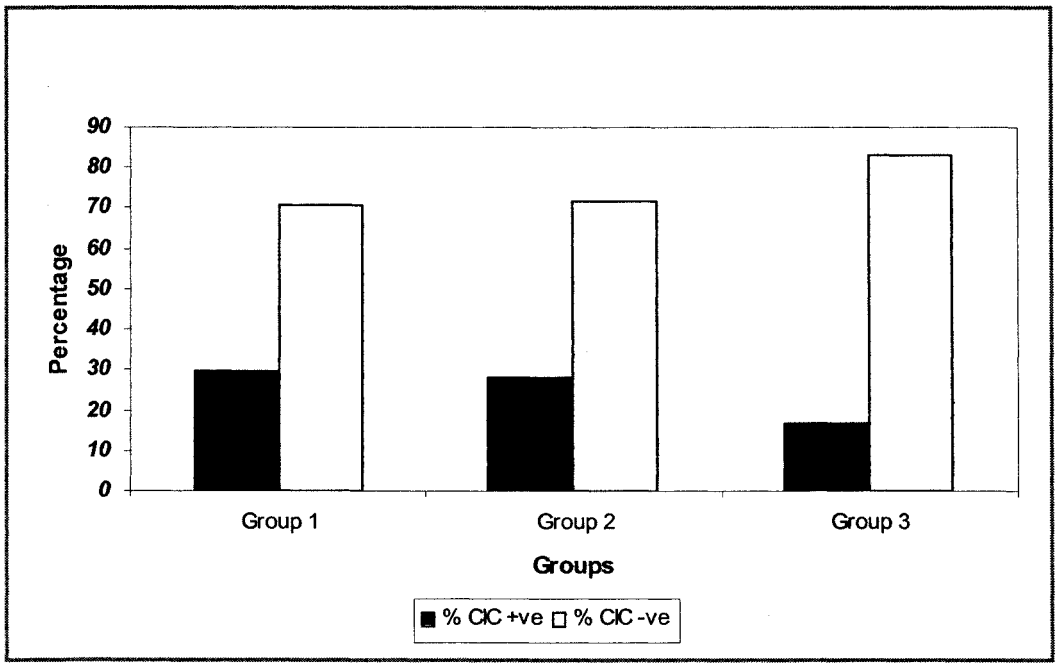

Figure 3.2: Results of CIC detection by ELISA test. The percentage of positive and negative individuals for BDV CIC is shown for the patient and control groups.

The patient group (group 1) and the hospital control group (Group 2) had a similar proportion of positive results (29.5 % and 28.1 % respectively). Significantly lower number of people in the blood donor group (group 3) were positive for CIC (χ^2 = 9.003 p= 0.011).

Comparison of mean positive OD on the CIC and Ag assays

The mean OD for both the CIC and Ag ELISA for the three tests are shown in figure 3.3.

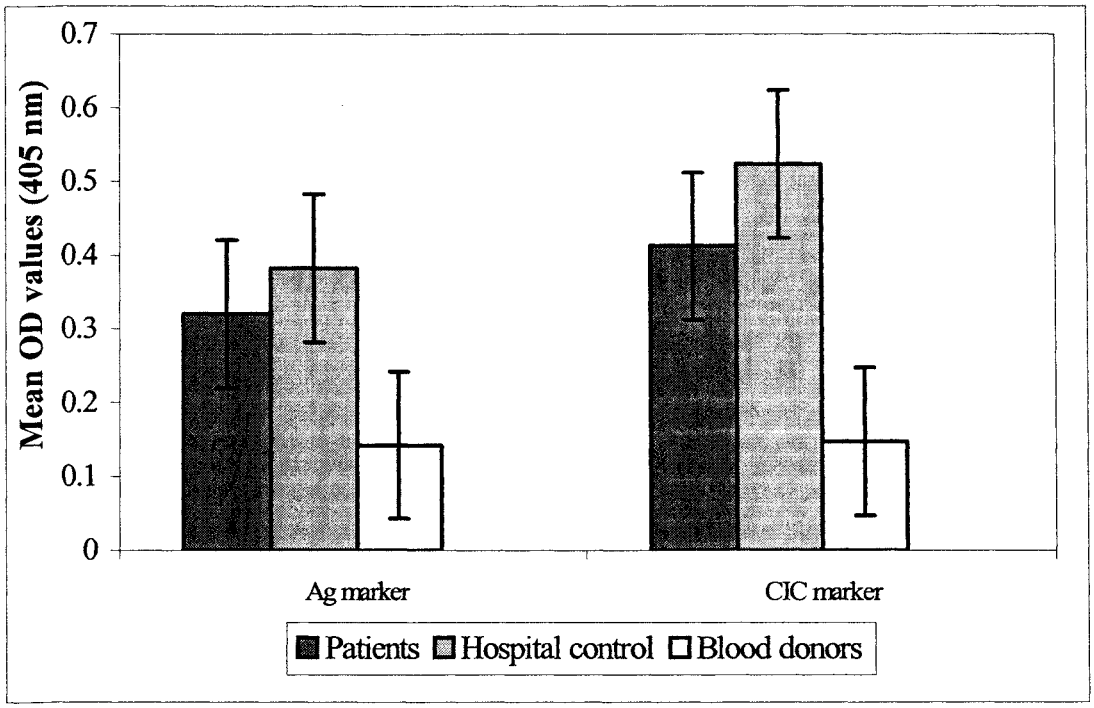

Figure 3.3: Comparison of mean OD for both CIC and Ag markers. The bars represent the mean positive optical density value for CIC and BDV Ag in the patients (group 1), hospital control (group 2) and blood donor (group 3) groups tested. The errors bars represent 95 % confidence limits. Ag = antigen; Ab = antibody.

The mean OD for group 1 and group 2 was significantly higher (0.32 and 0.412 for the Ag; 0.382 and 0.523 for CIC respectively) than group 3 (0.143 for the Ag and 0.148 for CIC) suggesting that there was more BDV antigen in the first two groups

of individuals as compared to individuals in blood donor group ($\chi^2 = 4.303$ p= 0.116).

3.2.1.3. Detection of anti-BDV IgG in serum

This assay measures anti-BDV IgG in plasma/serum. Briefly BDV antigen was bound to specific anti-BDV monoclonal antibody which had been coated onto an ELISA plate (methods section 2.3.1.). Test sera were incubated with the antigen and the binding of AP conjugated anti-human IgG to the antibody/BDV/antibody complex indicates that antibodies from tests or controls are present. Therefore, they will have been exposed to BDV although not necessarily infected at the current time. The colour obtained in the assay was therefore proportional to the amount of anti-BDV IgG and is shown as OD values (see appendices II and III). The results for the three test groups are shown in figure 3.4.

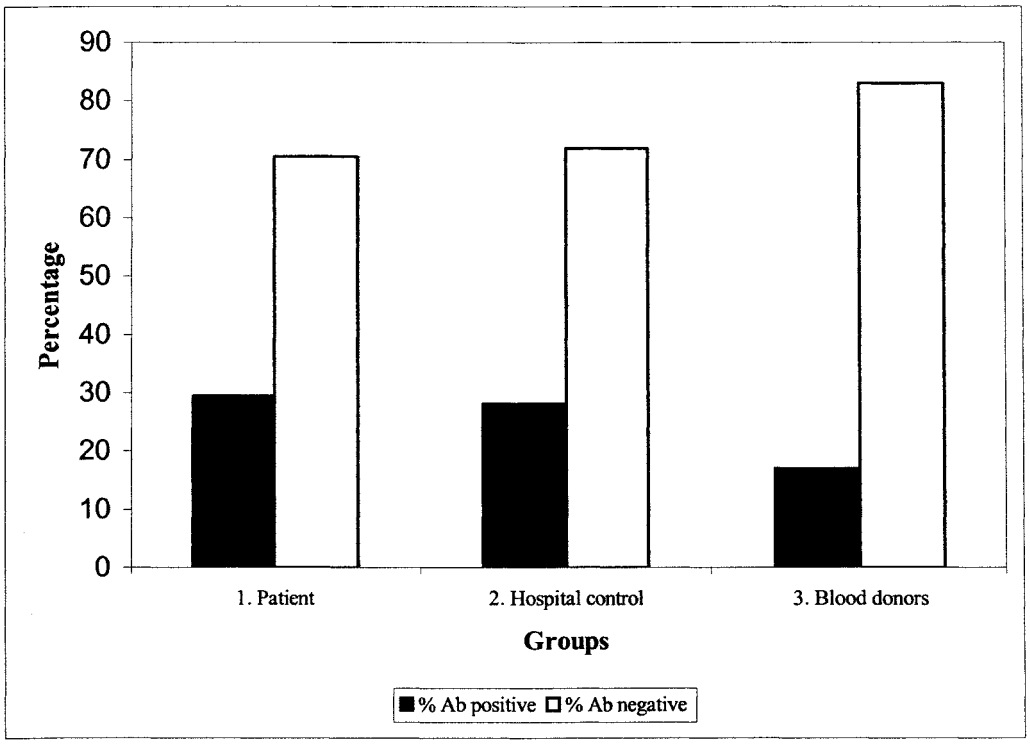

Figure 3.4: Results of anti-BDV IgG test by ELISA. The bars represent the percentage of presence/absence of anti-BDV antibodies in psychiatric patients (group 1), hospital controls (group 2) and blood donors (group 3), similar to those found in CIC data. Ab = antibodies

Group 1 (29.5 %) and group 2 (28.1 %) individuals were significantly more likely to have anti-BDV antibodies as compared to normal blood donors (group 3) which were significantly less likely to have anti-BDV antibodies ($\chi^2 = 9.00$; $p = 0.01$).

3.2.1.4. Summary of triplet ELISA data

The overall results for BDV Ag (Ag), anti-BDV IgG (Ab) and circulating BDV immunecomplexes (CIC) are shown on table 3.1. The overall result was only scored as ELISA positive if an individual was positive for antigen alone or for at least two of the three immunomarkers (Ag and CIC; Ag and Ab; CIC and Ab), as single positive CIC marker rarely occurred and the presence of anti-BDV IgG could be interpreted as cleared infection rather than current infection.

	N.	+ Ag	- Ag	+ Ab	- Ab	+ CIC	- CIC	**Overall ELISA**
Group 1	95	14 14.7 %	81 85.3 %	28 29.47 %	67 70.53 %	28 29.5 %	67 70.53 %	28 29.47 %
Group 2	32	6 18.75 %	26 81.25 %	9 28.13 %	23 71.87 %	9 28.1 %	23 71.87 %	9 28.13 %
Group 3	401	27 6.7 %	374 93.3 %	68 16.96 %	333 83.04 %	68 16.96 %	333 16.96 %	68 16.96 %

Table 3.1: The human triplet ELISA data. The number and percentage of positive/negative results for BDV antigen, anti-BDV antibody and CIC is shown. A positive ELISA was only scored if a serum sample was positive with an OD greater than 0.1. Only individuals that scored positive for antigen alone or for at least two of the three tests, (e.g. Ag, Ag and Ab, Ag and CIC or Ag and CIC) were counted as positive in the overall result for the triplet ELISA. Samples outside the range of 95 % confidence interval for the mean, after repeated testing, were scored negative. N = number, Ag = antigen, Ab = antibody, - = negative, + = positive.

Based on the overall results for the triplet ELISA, individuals with mood disorders (group 1) and the hospital control (group 2) were significantly more likely to be BDV positive than normal control (group 3) ($\chi^2 = 8.418$ $p= 0.015$).

3.2.1.5. BDV seroprevalence by ELISA in mood disorder patients subdivided into DMS-4 criteria

To determine whether BDV infection was associated with any particular clinical state, the mood disorder patient group was further subdivided into the following groups: Major Depressive disorder (MDD), Bipolar I rapid cyclers (BPIRC), Bipolar I not-rapid cyclers (BPInotRC), Bipolar II rapid cyclers (BPIIRC), Bipolar II not-rapid cyclers (BPIInotRC) according to the International criteria of mental diseases. Figure 3.5 shows the different categories and the results for each of them.

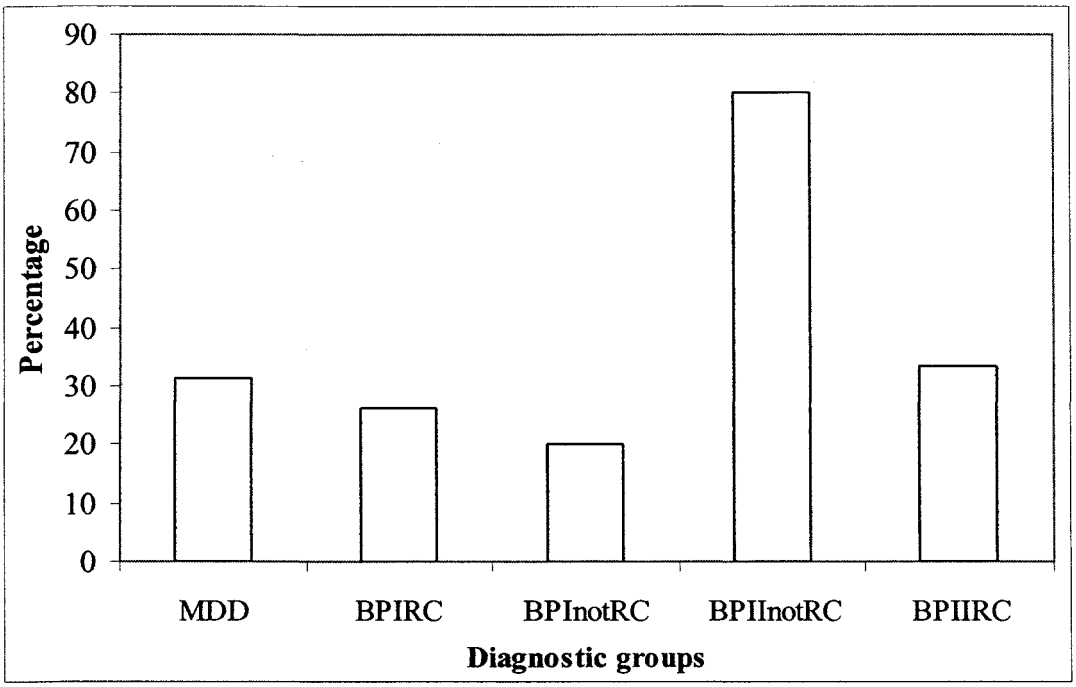

Figure 3.5: Seroprevalence of BDV in patients (group 1). The bars represent the results of the triplet ELISA tests for patient group analysed by mental disease criteria. Major depressive disorder (MDD, n = 11), bipolar type I rapid cyclers (BPIRC, n = 5), bipolar type I not rapid cyclers (BPInotRC, n = 6), bipolar type II rapid cyclers (BPIIRC, n = 4) and bipolar type II not rapid cyclers (BPIInotRC, n = 2).

In most cases 20 and 33 % of individuals were seropositive for BDV whilst 80 % of those individuals with BPII non RC were positive for BDV.

3.2.1.6. Age and sex distribution of BDV infection

Samples for this study were selected randomly and thus to determine whether BDV infection was associated with any particular age group the pattern of seropositivity was analysed and the results are shown in figure 3.6.

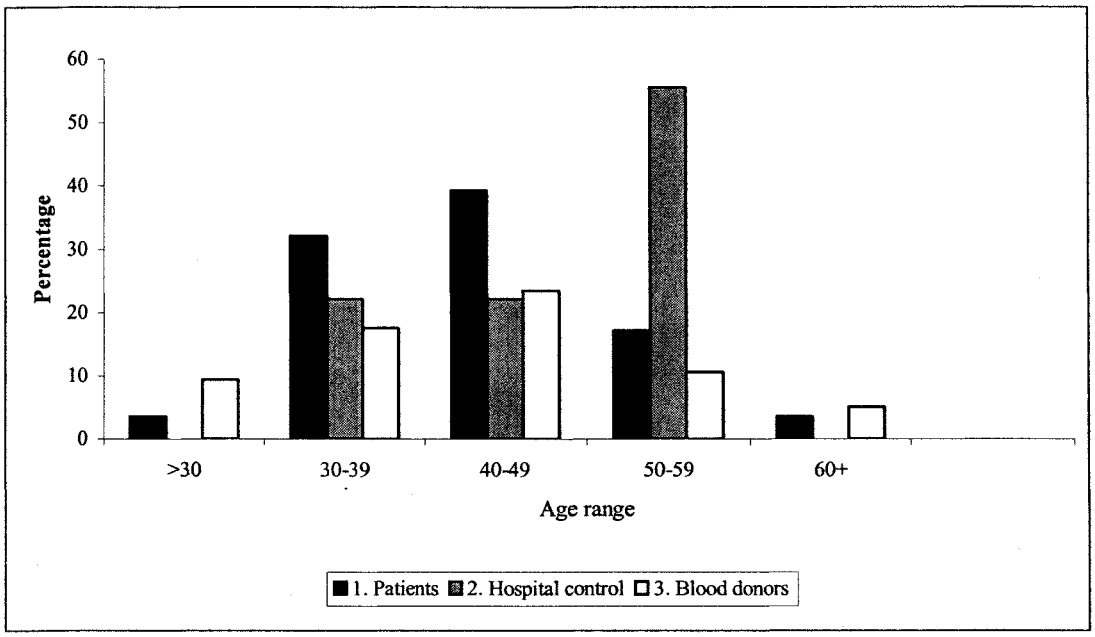

Figure 3.6: Seroprevalence of BDV by age within groups. The bars represent the percentage positive of BDV infection as detected by the triplet ELISA tests and the relationship with different age range within the three groups tested.

The results show that there were differences in the proportion of infected individuals in different groups. The majority of positive mood disorder patients (Group 1) and blood donors (group 3) were in the age range 40 to 49 (39 % and 23 % respectively) whilst in the hospital control group (group 2) the highest proportion of positive individuals was found in the age range 50 to 59 (56 %). Samples from individuals of less than 30 years old and greater than 60 years old were the least likely to test positive in all three groups (between 0 and 9 %). For the patient group, 3.57 % of individuals did not disclose their age range. For the hospital control (group 2) no individuals over the age of 59 or under the age of 30 were recruited.

In reference to gender, there were 48 female and 45 males in the mood disorder group (2 individuals were not determined), 15 male and 17 female in group 2 and 213 female and 188 male in group 3. Figure 3.7 shows the data for the number of affected people in relation to their gender.

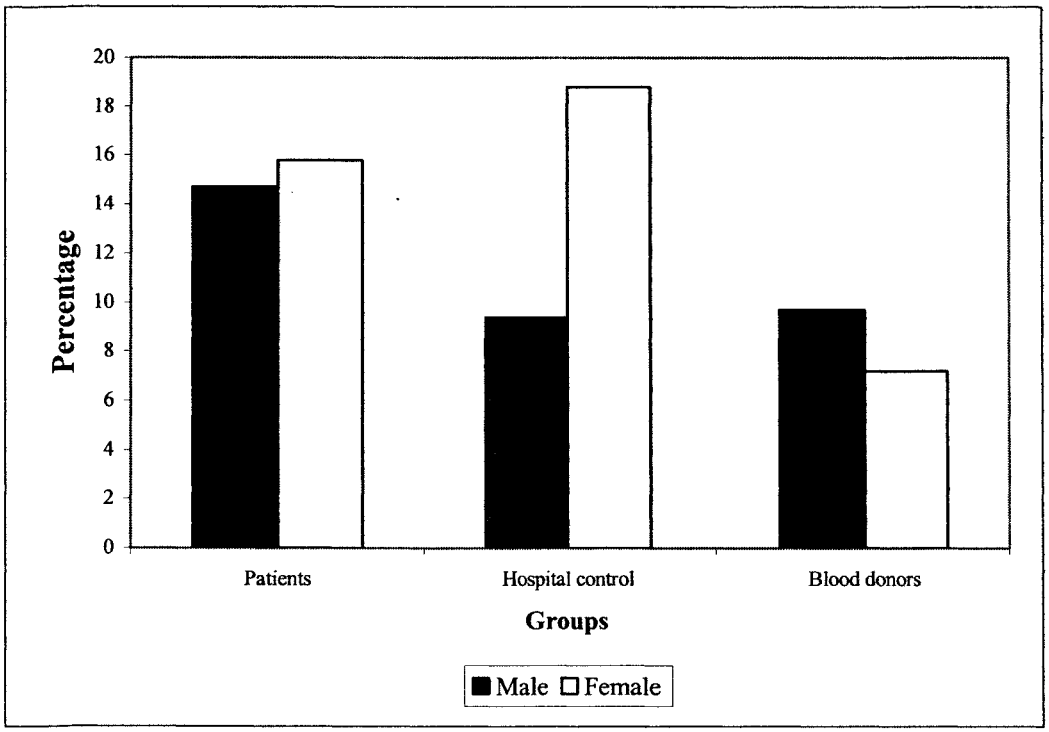

Figure 3.7: Percentage positive results for BDV positive individuals in the three tested groups as analysed by sex.

Group 1 (patients) have a total of 14/48 (14.7 %) positive males and 12/45 (15.8 %) positive females. Within this group, sex of 2 members was not specified. Group 2 (hospital control) have a total of 3/15 (9.4 %) positive males and 6/17 (18.8 %) positive female; lastly, group 3 (blood donors) have a total of 39/188 (9.7 %) positive males and 29/213 (7.2 %) positive female. These results show that there were more

affected women in group 1 (patients) and group 2 (hospital control) than in group 3(blood donors). The difference in infection levels of both female and male in groups 1 and 3 was not significantly different (χ^2= 0.166; p=0.684; χ^2= 0.579 p=0.447 respectively) whilst in group 2 significantly more males were infected (χ^2 = 4.153; p=0.042). If considering all these groups together, there was no significant difference in infection between sexes within the three groups, the difference was not significant (χ^2 = 0.043; p=0.836).

3.2.2. Detection of antibodies to BDV p40 and p24 proteins in human sera by Immunofluorescence assay (IFA)

A total of 538 sera from mood disorder patients (group 1), volunteer hospital workers (group 2) and volunteer blood donors (group 3) (see 3.1.1) were tested for anti-BDV p40 and p24 antibodies by IFA.

Samples were judged positive or negative according to the distinct pattern of nuclear fluorescence as observed with FITC labelled anti-human conjugated and TRITC labelled anti-mouse conjugated. An example of the fluorescent pattern in controls and tests is shown in figure 3.8.

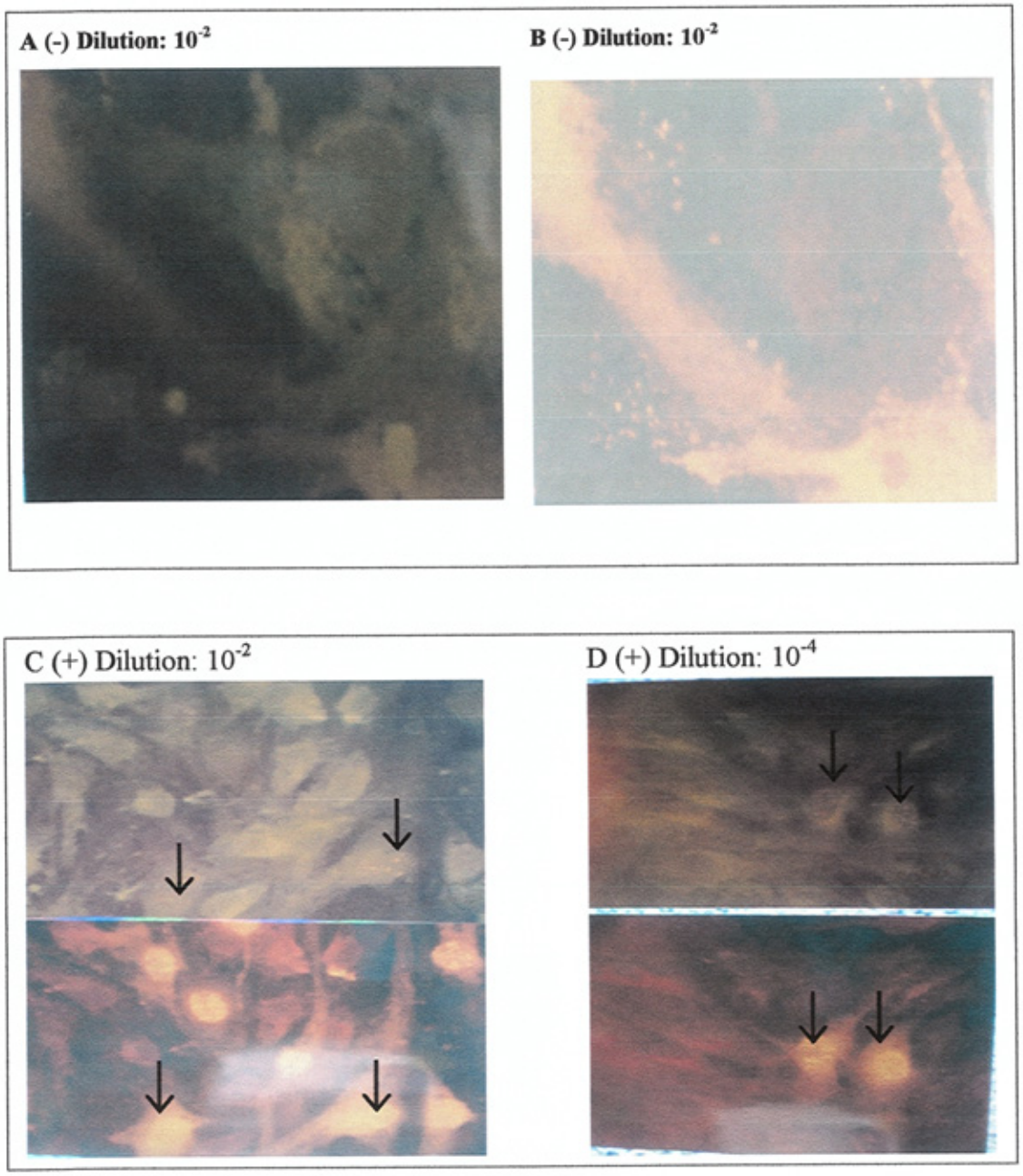

Figure 3.8: Results of IFA test for p24/40. BDV infected to uninfected OL cells immunohistochemically stained with fluorochromes (anti-human FITC and anti-mouse TRITC) and viewed under x40. A & B: negative control serum tested at a dilution of 1:20. No nuclear or cytoplasmic fluorescence is apparent. C & D: Pictures were from the same positive test sample at two different dilutions. Although the photographs are not very clear, both top and bottom show exactly the same nuclear pattern with the FITC and TRITC as indicated by the arrows.

The absolute results of the IFA assay was somewhat different from that of the triplet ELISA for the three test groups and is shown in figure 3.9. In spite of this, the overall pattern of group 1 and 2 having more positives than group 3 was similar to that of the ELISA.

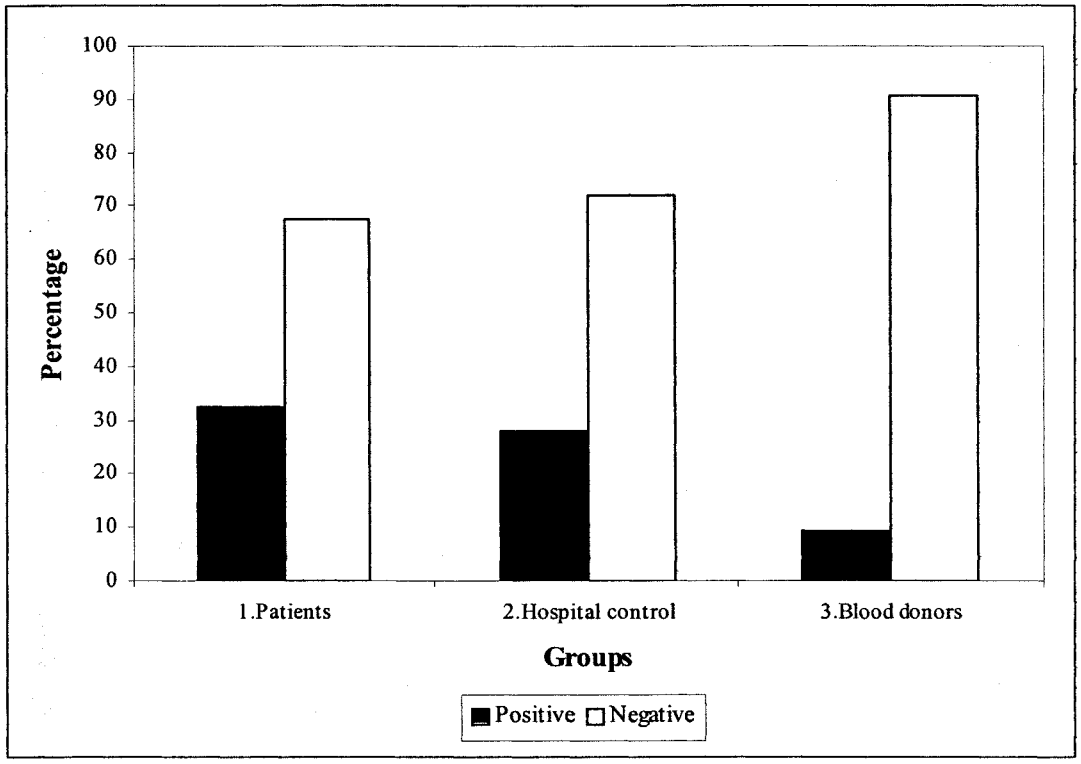

Figure 3.9: Seroprevalence of BDV by IFA. The bars represent the percentage of positive /negative results with the IFA test for group 1 (patients), group 2 (hospital control) and group 3 (blood donors).

About 33 % of mood disorder patients were seropositive for BDV by IFA. The hospital control (Group 2) was positive in 28 % of cases and the blood donors (Group 3) were positive in about 9 %. Thus, significantly more individuals in group 1 and group 2 were positive for BDV as compared to group 3 ($\chi^2 = 38.789$, P =

0.000). Full details are shown in table 3.2, which also gives a comparison between both serological tests (IFA and ELISA).

3.2.3. Overall BDV seroprevalence in patients and controls

The overall results for the seroprevalence of BDV in humans are shown in table 3.2.

Group	N.	IFA	ELISA
1. Patients	95	31 (32.63 %)	28 (29.47 %)
2. Hospital control	32	9 (28.13 %)	9 (28.13 %)
3. Blood donors	401	37 (9.23 %)	68 (16.96 %)

Table 3.2: Data from both serological analysis (IFA and ELISA). The number of positive samples as well as their respective percentages is shown. Note that hospital controls are made of individuals in direct contact with psychiatric patients (e.g. psychiatrist, nurses and social workers). N. = number

Slightly more mood disorder patients (about 4 %) were seropositive for BDV by IFA than by ELISA. However, for the blood donor group, a considerable higher percentage of seropositivity was obtained by ELISA test (8 %) than by IFA. The hospital control (Group 2) gave the same score by both methods (9 %).

3.3. Detection of BDV markers in horses by serological analysis

3.3.1. Detection of BDV infection in horses by the triplet ELISA test

The triplet ELISA test was used to screen 274 samples collected from horses for BDV. Among this number 78 were from the Newcastle region (group 1) and 196 were from a national surveillance program (group 2).

3.3.1.1. Detection of BDV antigens

Similar to the human assay, BDV antigens were detected by means of a direct ELISA (section 3.2). The results for the antigen assay in horses are shown in figure 3.10 and they indicate that the number of positive horses from the group 2 (national surveillance) was higher than those from group1 (Newcastle region).

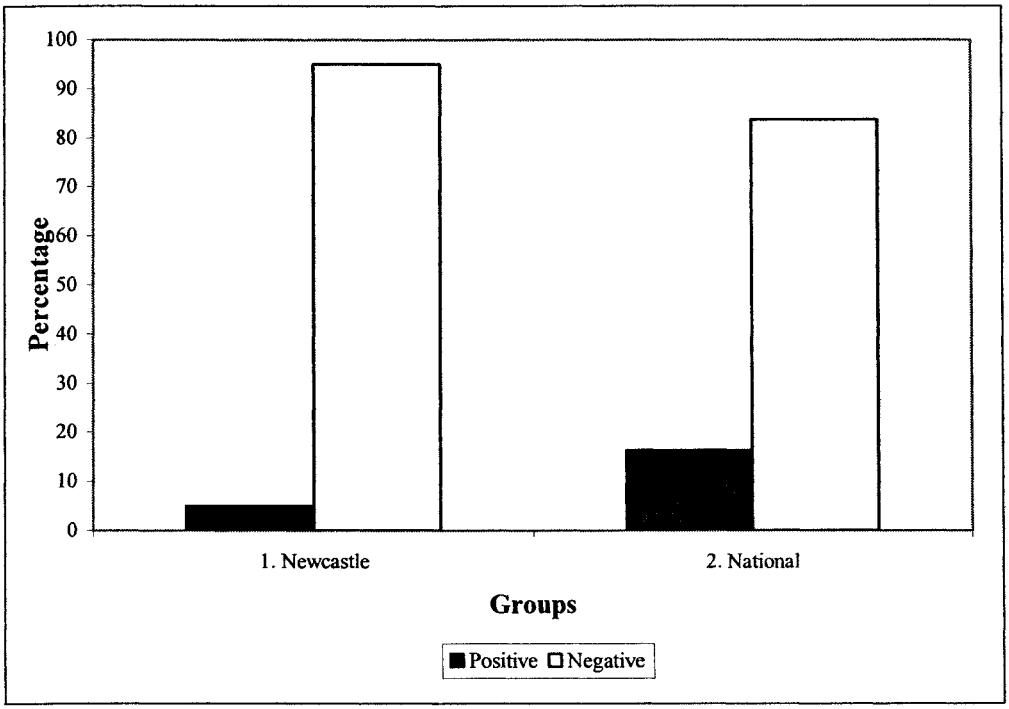

Figure 3.10: Results of BDV antigen detection by ELISA test in sample test of horses. The bars represent the percentage of horses positive for BDV antigen with the antigen assay in the triplet ELISA.

In this assay only 4 out of 78 horse samples (5 %) from the Newcastle region (group 1) were positive for BDV antigen test. In contrast significantly more horses (16 %; 36 out of 274) from the national surveillance programme (group 2) were positive for BDV (χ^2 =6.31; p=0.013). Taking both groups as a whole (regional and national) 36/274 horse (13.14 %) were positive for BDV antigen.

3.3.1.2. Detection of anti BDV IgG in serum of equine samples

Antibodies to BDV in samples were detected by screening the samples with antigens as described earlier (section 2.2.1.) and it was recorded in figure 3.11. Individual details are shown in appendices III-a, III-b.

Data for anti-BDV IgG in the horses tested in this study is shown in figure 3.11.

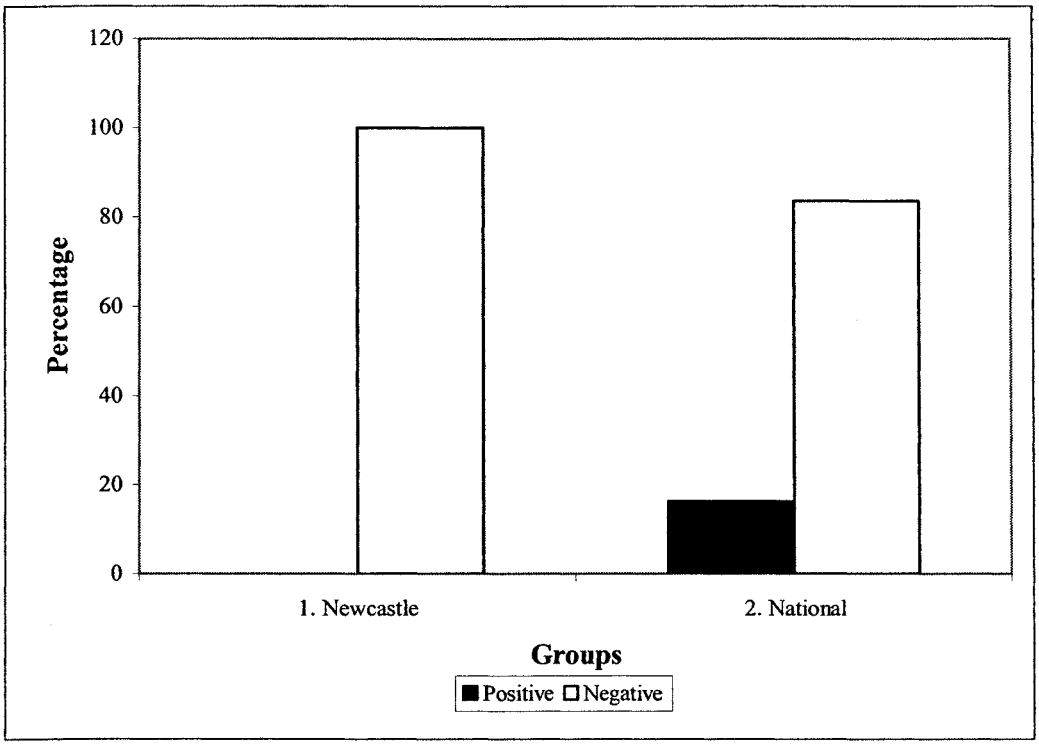

Figure 3.11: Results for the anti-BDV IgG test with the triplet ELISA in horse samples. The bars represent the percentage of positive/negative horses for the antibody test. Note that there were no positive results from Newcastle region for the anti-BDV antibody test.

None of the horses from Newcastle region were positive, while ~ 16 % of horses from the national surveillance were positive for anti-BDV IgG (χ^2 =14.419; p< 0.001).

3.3.1.3. Detection of BDV CIC

Immune-complexes were detected by the BDV CIC ELISA. The results for the two equine groups are shown in figure 3.12.

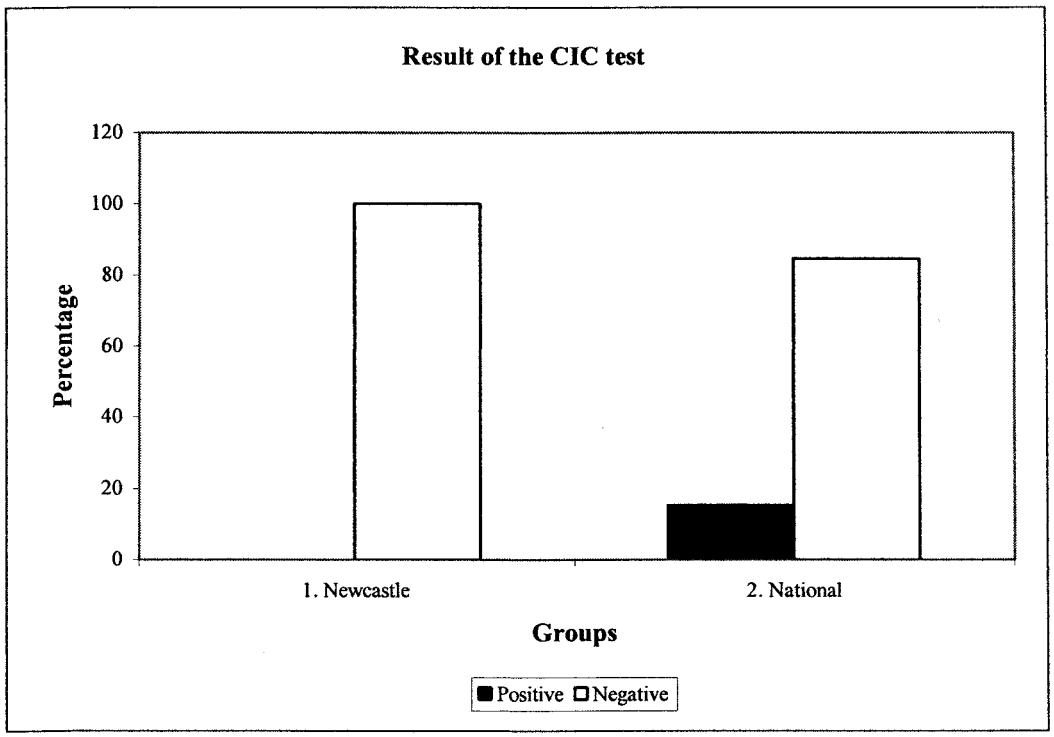

Figure 3.12: Horse population with/without BDV circulating immune complexes. None of the horses from Newcastle region (group 1) was positive for the CIC test..

May be unsurprisingly when considering the anti-BDV IgG results that none of the group 1 horses were positive for CIC whilst ~ 15 % of horses from group 2 were positive for BDV (χ^2 =13.407; p<0.001).

3.3.1.4. Distribution of BDV positive horse samples by region

A summary of the results for BDV seroprevalence in horses by the triplet ELISA is shown in table 3.3 and figure 3.13. This data also includes a description of the areas where the samples were collected.

Results for the triplet ELISA tests analysed by area

Origin	Ag	Ab	CIC	*Number positive	% Positive
Newcastle	4	0	0	4/78	5
Berks	1	3	1	1/21	4.7
Gloucester	5	4	4	5/7	74.4
Kent	0	1	1	1/3	33.3
Leicester	1	1	1	1/3	33.3
Oxon	1	0	0	1/2	22.2
Powys	1	1	1	1/1	100
Rep. Ireland	17	16	16	17/49	34.7
Suffolk	5	5	4	5/76	6.6
Sussex	1	1	1	1/1	100
Yorkshire	0	0	1	0/2	22.2
**Other	0	0	0	0/31	0
Total	**36**	**32**	**30**	**37/274**	**13.5**

Table 3.3: Results of the triplet ELISA test in horses with respect to the number of samples tested and their origin. * Positivity was based on the presence of antigen alone, or for at least two of the three immunomarkers (Ag and CIC; Ag and Ab; CIC and Ab). Samples that were only positive for antibodies were considered negative as this may indicate past exposure to the virus and not current infection. **In addition a number of extra samples came from Cheshire (1), Cornwall (4), Denbigh (2), Denmark (1), Dorset (1), Essex (1), Hampshire (1), Hants (1), Israel (1), Lincs (1), N. Yorks (3), Norfolk (1), Oxfordshire (2), Shrops (1), Lancs (1), W. Midland (2), W. Sussex (2), and Wilts (1). There was no data for 4 samples.

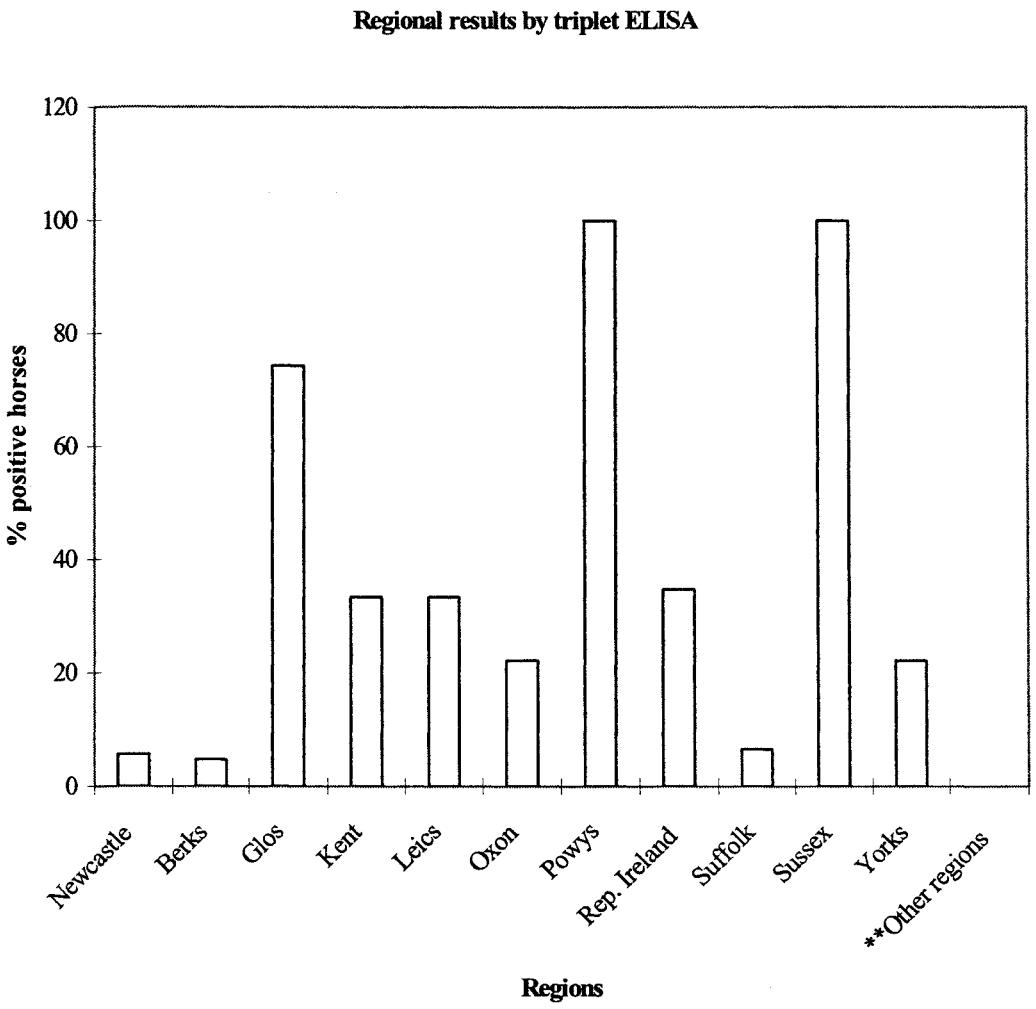

Figure 3.13: Regional results by triplet ELISA (for more details see table 3.3). *Other was 18 regions which had only negative samples.

There was a wide variation in seropositivity within areas. The highest proportion of seroprevalence was Powys and Sussex (100 %) although only one horse was tested in each of these regions. Gloucester was the second most affected area (75 %) and it was interesting to note that 34.7% (17/49) of the horses tested from the Republic of Ireland were positive.

3.3.2. Detection of antibodies to BDV p40 and p24 proteins in horses by immunofluorescence assay.

A total of 274 horses were tested for anti-BDV p40 and p24 antibodies by IFA. Samples were judged positive or negative according to either the distinct pattern of nuclear fluorescence as observed with FITC labelled anti-human conjugated and TRITC labelled anti-mouse conjugated. Endpoint titrations were achieved at either 1:40 or 1:80 dilutions. BDV antibody profiles in horses were similar to that of the humans reported earlier. Note that there was no clinical evidence of neurological or behavioural disease in any of the horses tested. An example of the fluorescent pattern is shown in figure 3.14.

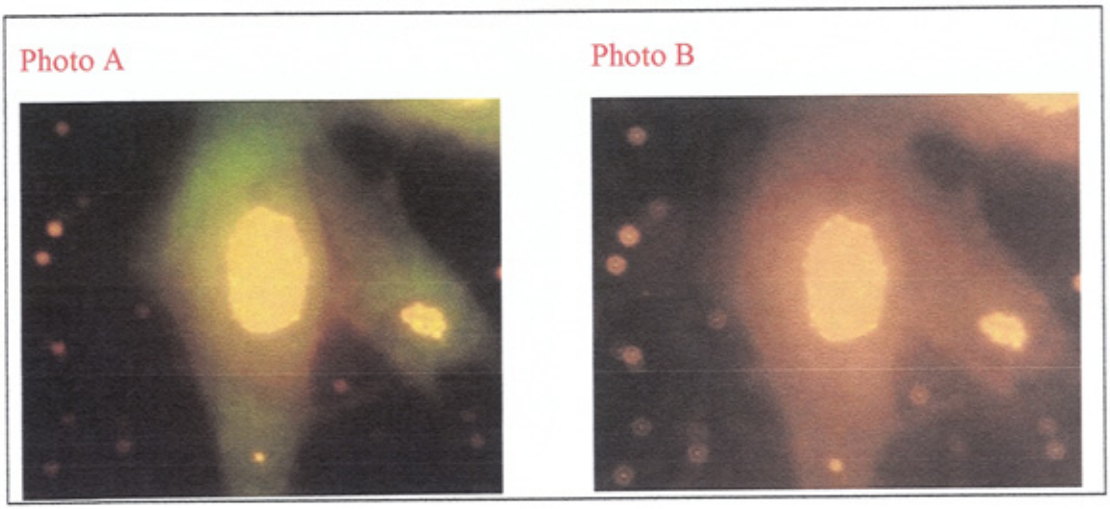

Figure 3.14: (A and B) Immunofluorescence test with YRS cells. The photographs show the same antigen patterns (intranuclear fluorescence) with both fluorescein (FITC-A) and rhodamine (TRITC-B) in a BDV infected horse from Newcastle region (Horse 12) with a 10^{-4} dilution.

Results of the IFA test on horse samples are shown in figure 3.15.

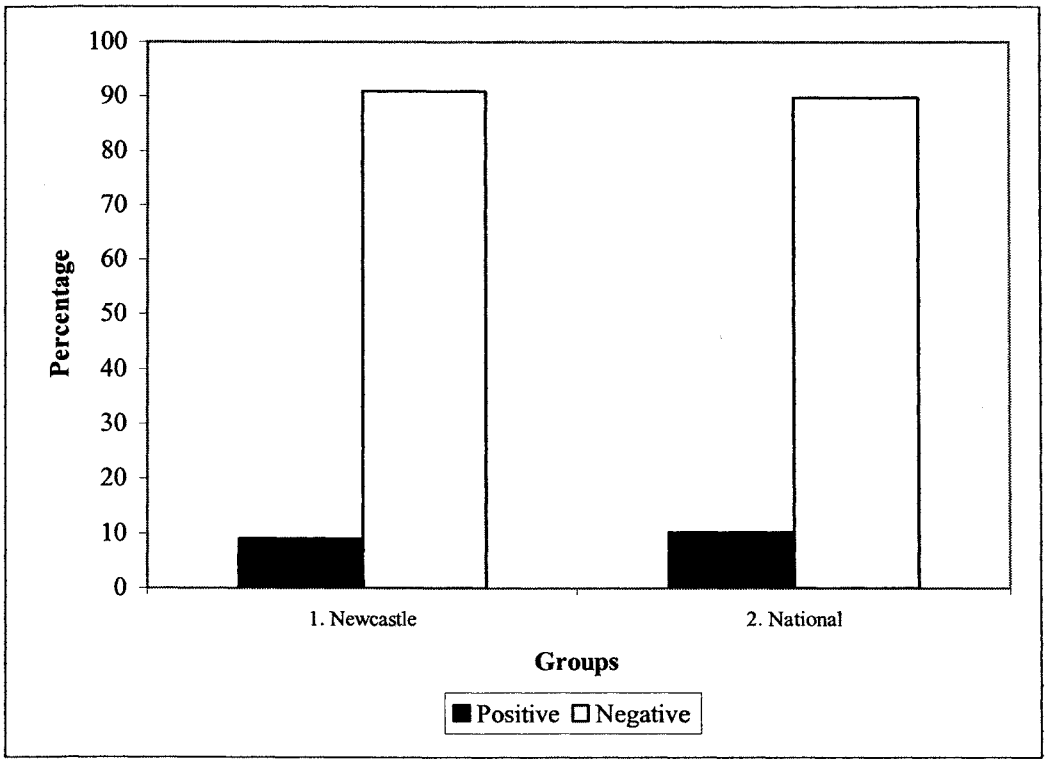

Figure 3.15: Results from IFA analysis. The bars represent the percentage of horse samples, from Newcastle and from the National surveillance program, positive for anti-BDV IgG by IFA analysis.

Nine percent of horses in the Newcastle region (7 out of 78 horses) and 10 % of the National surveillance program horses (20 out of 196) were positive by IFA (χ^2=0.095; p=0.758).

3.3.3. Summary of equine BDV seroprevalence

A summary of the BDV seroprevalence in the equine samples tested is shown in table 3.4.

	TOTAL SAMPLES	**TOTAL POSITIVE**	**POSITIVE (%)**	**NEGATIVE (%)**
Newcastle	78	7	**8.97**	91.03
National surveillance	196	20	**10.21**	89.79
Total horses	**274**	**27**	**9.85**	**90.15**

Table 3.4: Summary of horse data for the serological analysis.

In general BDV positivity of horse samples originating from Newcastle were consistently lower than the national surveillance. The results obtained from the IFA and ELISA were different with 9 % of the Newcastle group testing positive by IFA and only 5 % being positive by ELISA. In the national surveillance group the results were opposite with fewer positive cases being detected by IFA (~10 %) than ELISA (~13 %).

3.4. Analysis of BDV prevalence by molecular methods

3.4.1. Sensitivity and trial of RT-PCR

RNA from YRS/TL cells (1 x 10^5 ffu/ml) was extracted and quantified by a colorimetric method (section 2.4.3.) with 256 µg RNA being obtained. This was serially diluted in 1 x TE buffer to give a fold dilution of 1 pg l^{-1}. The extracted-RNA was then reverse transcribed and amplified by two different methods. The results of these titration experiments are shown in Figure 3.16.

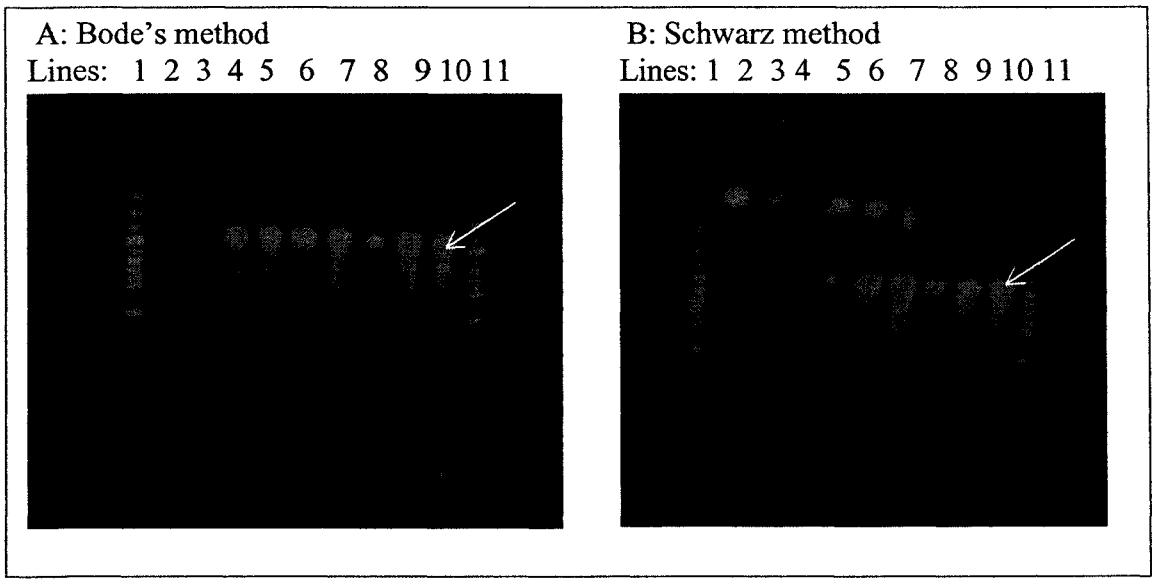

Figure 3.16: Analysis of RT-PCR sensitivity by two methods. The molecules amplified were BDV p40-specific RNA, which were serially diluted 10-fold. This RNA template added to the reactions were (starting from left): 10 µg and no primers (lane 2), no template (lane 3), 1 pg (lane 4), 1 ng (lanes 5), 1 µg (lane 6), 10 µg (lane 7), Lane 8, 9 and 10 were repeats of the previous 3 lanes, Markers were on the first and eleven lanes. The products were analysed on a 1.5 % ethidium bromide-stained agarose gel. The position of 449 bp (gel A) and 441 bp (gel B) products are indicated by arrows.

Figure 3.16 also shows that the reaction without the hot start (Schwarz, picture B) has a higher ratio of primers and probes to the amount of target showing as a dense band of primer-dimmers on top of gel B. The use of the hot start prevented the detection of non-specific products, primer-dimmer formation and background at the same time increasing the sensitivity of detection of target as shown in gel A.

Optimal results were attained with the Qiagen master mix when the recommended concentrations and primers were used (methods section 2.4.5.) without major reaction adjustments. Not surprisingly, when comparing the results of the methods used (Bode and Schwarz) it seemed that the Bode method was more sensitive than that of the Schwarz method as after the second round seven out of the nine samples containing serially diluted BDV RNA molecules (from first round) were positive (figure 3.16-A). In contrast the Schwarz method was simpler to run (since it only required a single run) but the results were less sensitive than Bode's as only six of nine samples were positive for BDV p40 RNA molecules (figure 3.16-B). The positive samples generated a 449 bp product and it was assumed that the detection limit of the RT-PCR system was in the range of 1–10 RNA molecules since samples containing 10 total RNA molecules were positive.

Considering that high background due to non-specific products can block out specific signals during a reaction, accurate detection was managed after prolonged cycles.

The second round PCR increased detection to 10 fold, since 1 out of ten samples was not detected on the first round but all were detected on the second.

3.4.1.1. RT and nested PCR reactions in Bode's method

The reaction conditions for PCR products in both RT and nested PCR were tested and results are presented in figure 3.17. Reactions were performed with duplicate titration experiments using Ratpool (3 x 10^4 ffu/ml) as a template. The total RNA extracted was 248 μg/ml and viewed with agarose gel.

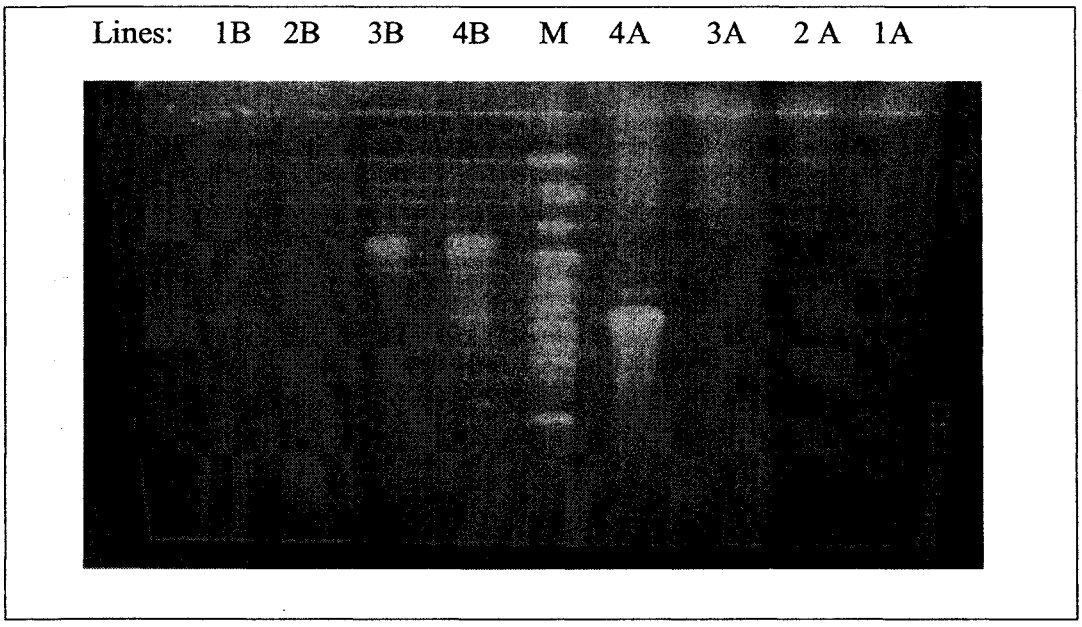

Figure 3.17: Amplification of nested RT-PCR analysis using the RT-PCR kit (Qiagen) and Bode's protocol. Key: Lane M in the middle of the gel contained the molecular marker. Right side of marker: Lane 1A to 4A had the product of the first round RT-PCR (Lane 1A with dH_2O, lane 2A with negative control (non infected OL cells), lane 3A with 1 ng of template and lane 4A with 1 μg template). The left side of the marker had the nested reaction or second round RT-PCR with the same templates.

Results of RT and nested PCR (figure 3.17) indicated that RT-PCR alone by Bode's method would not be as sensitive as the nested PCR. The gel shows clearly a 449 bp band corresponding to that of BDV p40 in lane 3 and 4 whereas on the first round there was only a 281 bp product. The RT-PCR also showed an accumulation of product as primer/dimmers (figure 3.17 on top and right of marker).
It was also shown that the nested RT-PCR using BDV RNA was negative when the reverse transcriptase was omitted during reaction indicating the absence of contaminated plasmid DNA (figure not shown).

3.4.1.2. The effect of Q-solution

To determine if secondary structures (i.e hairpin loops) were interfering with the amplification of PCR products Q-solution was added in an effort to resolve the melting behaviour of the DNA. Figure 3.18 shows that the specificity of amplification of the 441 bp PCR fragment was not improved by the addition of Q solution and therefore was not used in subsequent reactions.

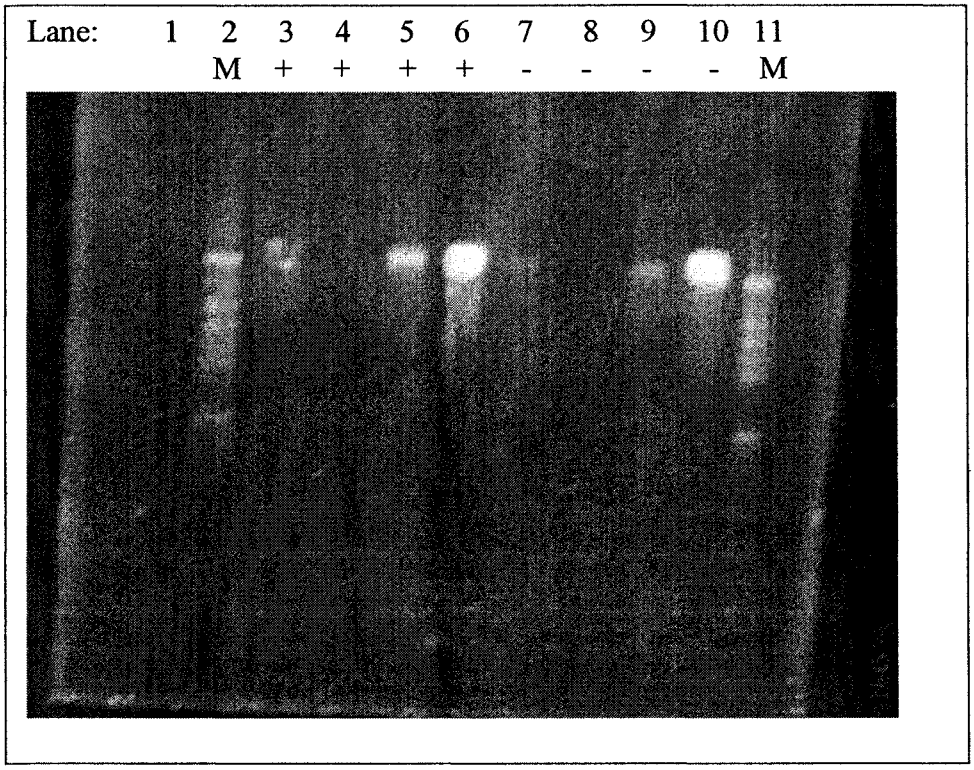

Figure 3.18: Effect of Q solution: A BDV 449 bp product from OL cells was amplified using Qiagen Kit in the presence (+) and absence (-) of Q solution. The product was amplified when Q solution was added to the solution and also amplified when the Q solution was not added in the reaction mixture. Results from duplicate nested RT- PCR amplifications are shown. Lanes 2 and 11 markers (100 bp marker with 2 µl dye and 10 µl ladder), negative control on lane 1, lanes 3 and 7 contained 1 pg of tRNA from the OL/TL cells, lanes 4 and 8 had 2 pg of tRNA but no primers, lanes 5 and 9 had 2 pg and line 6 and 10 had 2 ng of RNA from the OL/TL cells.

For the gel electrophoresis 10 µl PCR products were mixed with 2 µl dye and run on a 2 % gel. This experiment also shows the effectiveness of the primers along with the Q solution and these results also show that 2 ng of the BDV p40 RNA could be used as a reliable standard to control the performance of the amplification system.

3.4.2. Viral RNA in serum and cells of the peripheral blood of infected humans

Patient and control peripheral blood cells and plasma were investigated for the presence of BDV-specific RNA. Serum was tested from those same EDTA-treated blood samples in which anti BDV antibodies had previously been detected. Positive results showed a band of 449 bp corresponding to that of the fragment from either the persistently infected YRS/TL or OL/TL cell lines. An example of a gel staining positive and negative results from patient and controls are shown in figure 3.19.

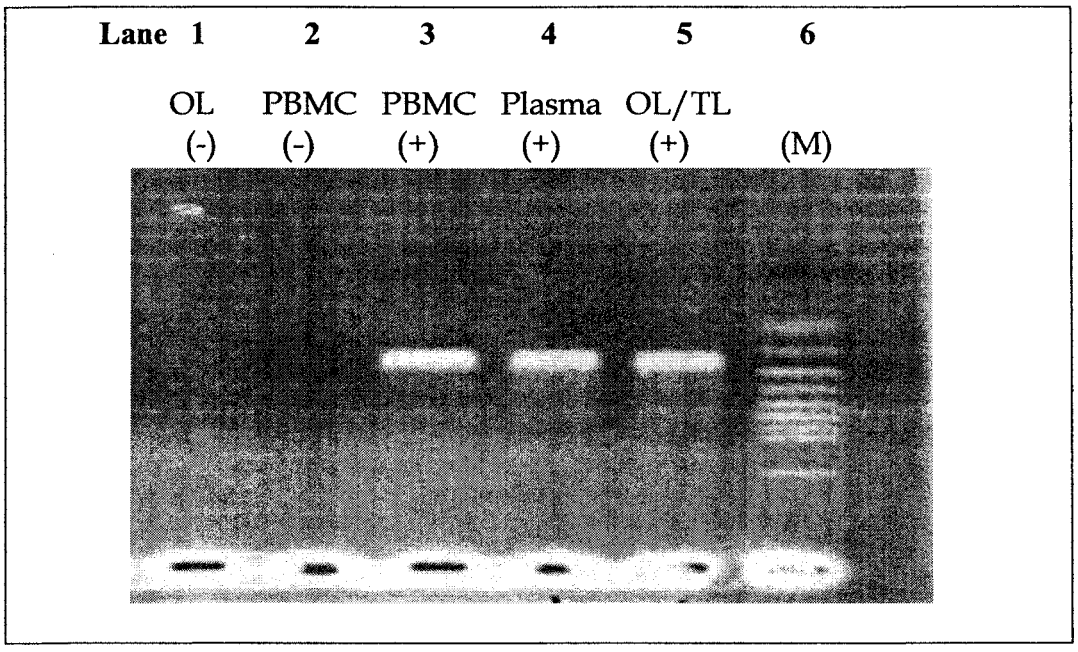

Figure 3.19: RT-nested-PCR reaction for the detection of BDV RNA. The results show an ethidium bromide stained bands. The templates starting from left: Lane 1 = negative control (OL cells); lane 2 = PBMC of patient 1; lane 3 and 4 = PBMC and plasma of patient 12; lane 5 = positive control (OL/TL) and lane 6 = 100 bp ladder (markers). (-) = negative, (+) = positive.

The PCR results for the three test groups are shown in figure 3.20.

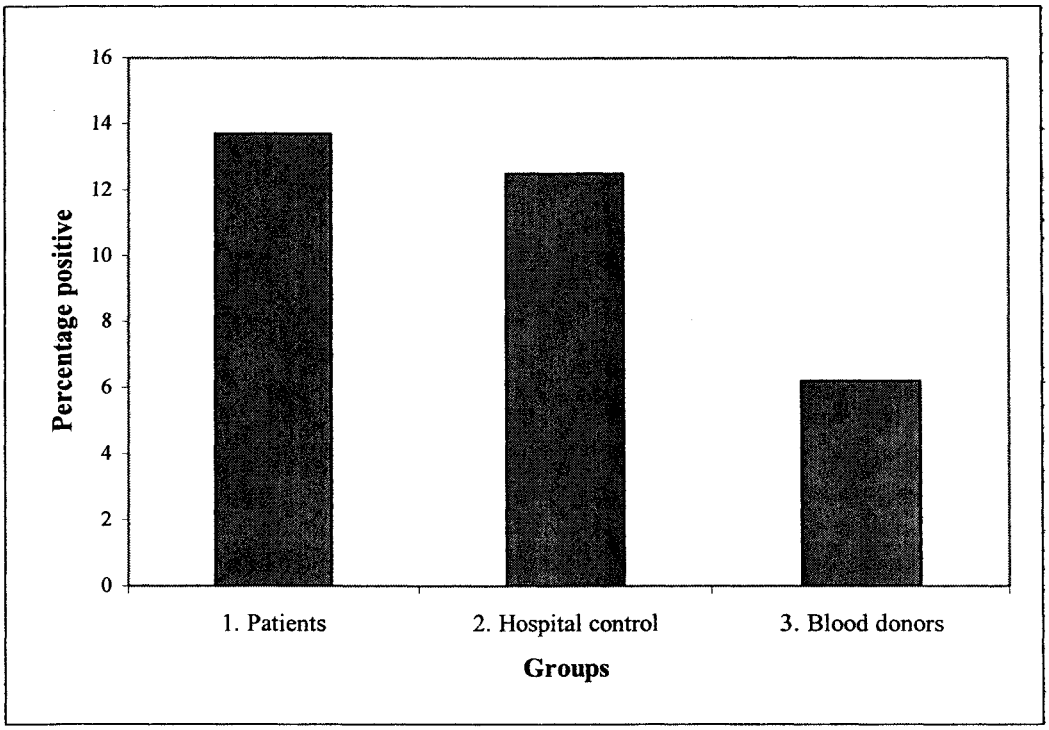

Figure 3.20: Results of BDV specific RNA in humans. The bars represent the percentage positive of BDV-specific RNA in human samples from group 1 (patients), group 2 (hospital control) and group 3 (blood donors).

BDV-specific RNA was detected in 13 out of 95 patients with mood disorders (group 1). Within this group, 8 out of 36 were patients with MDD, and 5 out of 59 patients suffered from bipolar disorders. BDV specific RNA was also detected in 4 out of 32 hospital control (group 2) and 25 out of 401 blood donors (group 3). Thus significantly more people in group 1 or group 2 were positive for BDV RNA ($\chi^2 =$ 6.783 $p = 0.034$) than group 3.

PBMCs and plasma prepared from patient number 2 (whose blood was used for the isolation of BDV as described in section 3.5) were subjected to RT nested PCR using primers to amplify DNA fragments with predicted sizes of 449 bp, corresponding to p40 BDV protein. RNA from BDV-infected OL cells (OL/TL) and uninfected (OL) were used as positive and negative controls, respectively. PBMCs of a negative patient were also analysed as seen in figure 3.20. PCR products were resolved by agarose gel electrophoresis and visualised by staining with ethidium bromide.

3.4.3. Viral RNA in cells of the peripheral blood/plasma of infected Horses

BDV-specific RNA was detected in peripheral blood cells of 22 horses by nested-PCR (figure 3.21). Peripheral blood cells of uninfected animals, negative control sample (YRB cells) as well as reagent control samples were all negative and YRS/TL cells tested positive for BDV p40-specific RNA (positive control).

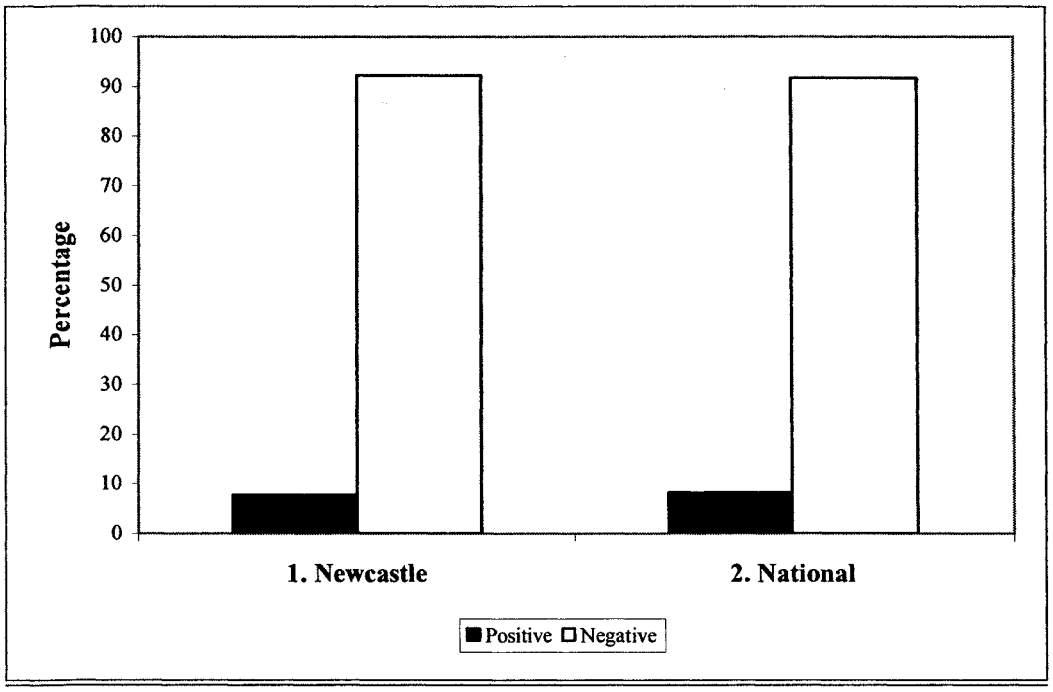

Figure 3.21: BDV RNA in plasma/serum of horses. The bars represent the percentage positive of BDV-specific RNA in the sera/plasma samples of horses from group 1 (Regional) and group 2 (National).

BDV RNA was detected in 6 out 78 animals from Newcastle (group 1) and 16 out 196 horses from national surveillance (group 2). Within the positive samples from group 2, 5 out of 7 (74.4 %) of the animals from Gloucester, 1 out of 3 (33.3 %) from Leicester and 9 out of 49 (18.4 %) from Republic of Ireland were RNA positive animals and this made the bulk of the positive animals. When relating to the results found with serological methods (sub-section 3.3.1.4 and table 3.3) it can be seen that for some regions the results were similar for both methods whilst other contrasted.

3.4.4. Sequence analysis of positive samples

Amplified fragments derived from horses and humans were sent to Qiagen sequence analysis services to determine the specificity of the PCR products obtained. The nucleotide sequences were determined and compared with the sequence of the BDV reference strain V (length = 8910). Sequence alignment and nucleotide changes for one example are shown in table 3.5. Results of sequence divergences to the reference strains ranged from 0.1 % to 5 % and are shown in appendix IV.

```
N. 2 : 1   caacacaaaggagcctacccagggttggccgttaatccaatctatagcctcatgtggatt 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
St. V: 677 caacacaaaggagcctacccagggttggccgttaatccaatctatagcctcatgtggatt 618

N. 2 : 61  aaacatctggagtagtgtagcagtctcaccatgggatggccggtttaaggctgccatcat 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
St. V: 617 aaacatctggagtagtgtagcagtctcaccatgggatggccggtttaaggctgccatcat 558

N. 2 : 121 agttttaaacctttcttgatctgctcggctcctgctttgatcttagacgacgatcctat 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
St. V: 557 agttttaaacctttcttgatctgctcggctcctgctttgatcttagacgacgatcctat 498

N. 2 : 181 cacaacccctattagtaatgagcaacaatggctgaagatagaggagatctccagctcggt 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
St. V: 497 cacaacccctattagtaatgagcaacaatggctgaagatagaggagatctccagctcggt 438

N. 2 : 241 gagatcacgctgcgtcgtcttttccccgtaaaacttcgcagtcttaacaacagtctgttc 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
St. V: 437 gagatcacgctgcgtcgtcttttccccgtaaaacttcgcagtcttaacaacagtctgttc 378

N. 2 : 301 tccacgcgtgacaggcgtcgacaggtaggattcacgaggcacccctccgtgaacaaacgc 360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
St. V: 377 tccacgcgtgacaggcgtcgacaggtaggattcacgaggcacccctccgtgaacaaacgc 318

N. 2 : 361 agcgtgcaagtcctgggattagcaaacatag 391
           ||||||| ||||||||||||||||||||||
St. V: 317 agcgtgc-agtcctgggattagcaaacatag 288
```

Table 3.5: Sequence alignment of positive BDV samples from a hospital patient number 2 (group 1) and BDV strain V. Matches are connected with a "|" symbol. Mismatches are opposed with a space. Gaps are introduced with a "-" symbol.

The sequence of a BDV isolated from a hospital patient (number 2) was compared to the complete genome of Borna disease virus strain V. The sequences alignment were analysed by NCBI-blast2 (matched in pairs) and showed 99 % similar identities (390/391) and 0 % of Gaps (1/391).

3.5. BDV isolation

The rationale for the isolation of BDV from a patient was based on the observation that BDV infectivity can be successfully rescued from patient PBMCs and plasma (Nakamura *et al.*, 2000). The positive control was Oligodendroglial (OL) cells infected with a preparation of 12-week-old rat brain which had previously been infected at birth with BDV strain V (ratpool) and the negative control was uninfected OL cells. In summary, samples involved in this experiment included one member of the patient group (Pte; Group 1) from the psychiatric hospital in Newcastle, one member of the hospital control group (C1; Group 2) and one from the blood donor group (C2; Group 3) as described in table 3.6.

	Patient (Pte) (n.2)*	Control 1 (C1) (n.64)*	Control 2 (C2) (n.124)*
Status	Chronically depressed	Healthy	Healthy
Diagnosis	MDD	Non	Non
Age	33	37	24
Sex	Male	Male	Female
Profession	Incapacitate	Psychiatrist	Secretary
Ag (ELISA)	++++ (1.057)	- (0.030)	- (0.033)
Ab (ELISA)	++++ (1.129)	++ (0.427)	- (0.064)
CIC (ELISA)	+++ (0.827)	+ (0.178)	- (0.082)
IFA	+++ ($2x10^4$ ffu)	+ ($1x10^2$ ffu)	-
RT-PCR	+	-	-
Specimen	Blood	Blood	Blood

Table 3.6: List of individuals used in BDV isolation. The table details results for the individual samples. Note that these were individuals previously tested by molecular and serological methods prior to the decision of co-cultivation. For comparison and interpretation of results, various cell lines expressing BDV were also tested (KFU2, W1H8, YRS/TL p90/56, OL/TL p55) as positive controls. Negative is represented by the sign – and positive by the sign +.

The morphology of BDV positive and BDV negative OL cells are shown in figure 3.22. These cells were passaged every 3 to 4 days for up to 70 days after inoculation and BDV antigen expression was monitored by IFA with monoclonal antibody to BDV p40 (Kfu2) and p24 (W1H8). RT-nested-PCR and Western Blotting were also employed after each single passage to test for BDV infection.

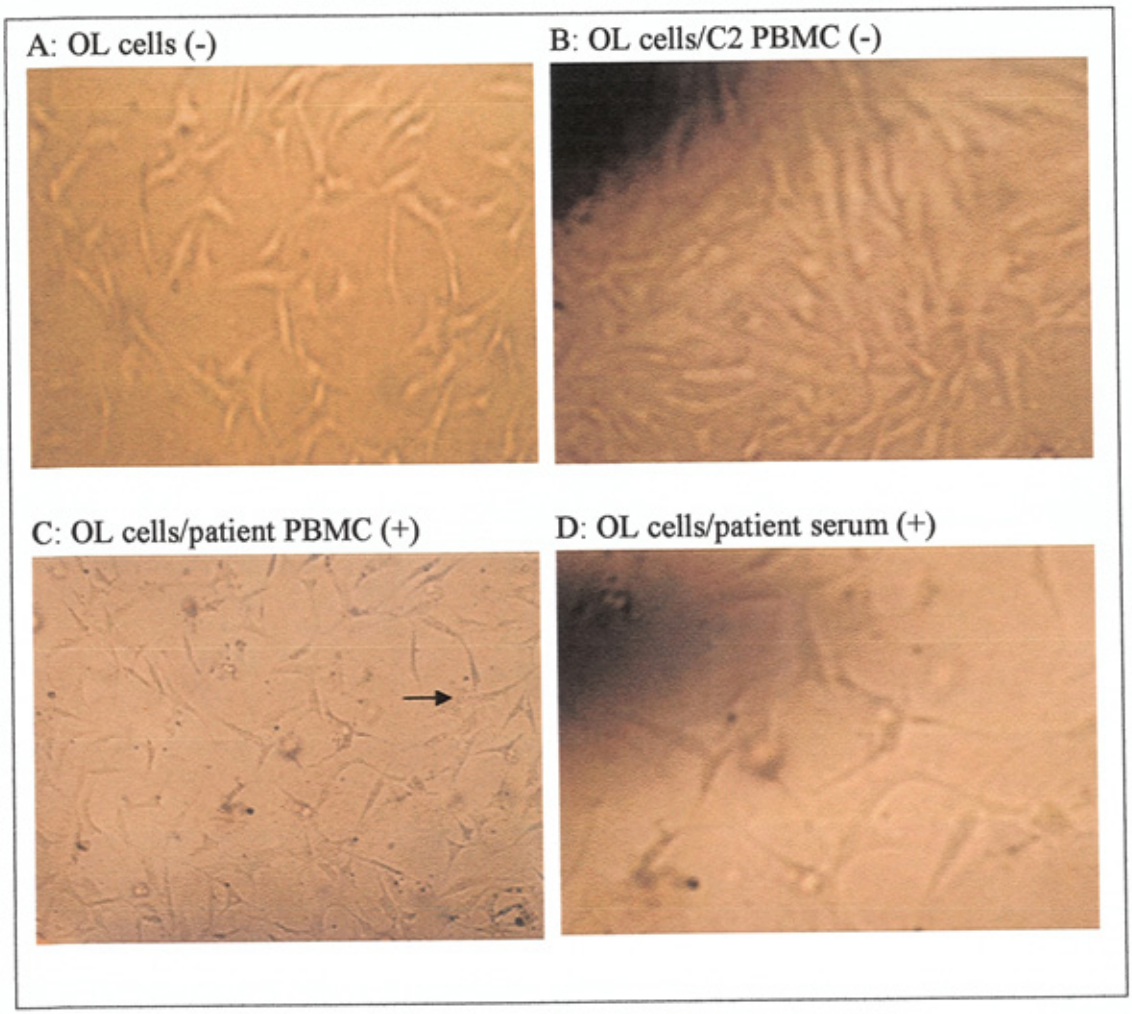

Figure 3.22: Morphology of normal and infected OL cells. A: normal cell culture of non-infected OL cells used as negative control; B: cell culture of OL cells cultivated with PBMC of C2 used as test samples; C and D: OL cells cultivated with patient PBMC and plasma respectively and also used as test samples. Arrow points to infected BDV cells with signs of cytopathic effect. C1 = control 1; C2 = control 2 (see table 3.6); (-) = non-infected cells; (+) = infected cells.

3.5.1. Detection of BDV in cells cultured with either PBMC or serum from Pte by IFA

The appearance of BDV antigen in the infected OL cells was monitored by IFA as described previously. BDV-positive cells were detected only in those OL cells inoculated with either patient (Pte) samples or ratpool as shown below (figure 3.23). An example of a negative cell can be found in section 3.2.2 and figure 3.8 (page 130).

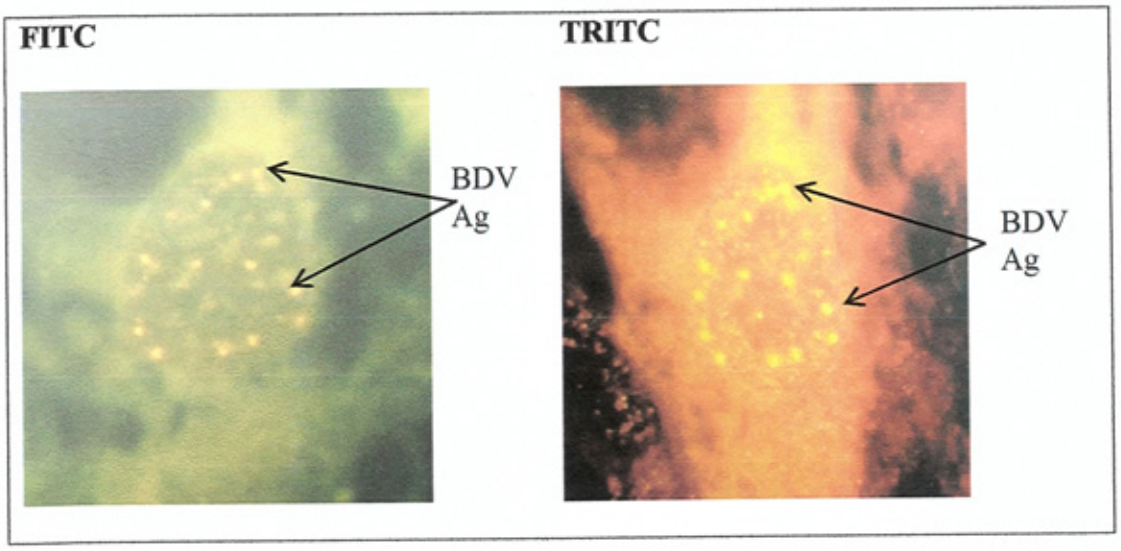

Figure 3.23: Localisation of BDV specific antigen in infected OL cells by IFA, test sample was derived from Pte. Passage 11: Intra-nuclear inclusions of varying size in single cells or few cells in nest-like areas of the monolayer are shown, stained with anti-human IgG FITC and anti-mouse IgG TRITC.

The figure above shows clearly that the sample was positive as the presence of solid fluorescence circular areas of various diameters in the nucleus of BDV positive cells were present. This staining was only occasionally observed in the cytoplasm

demonstrating that the nucleus was the major site of either virus replication or accumulation.

OL cells incubated with patient PBMCs were positive by IFA after 45 days (11 passages) with a titre of 20 whilst they were other OL cells positive 49 days (12 passages) after culture with plasma. Cells infected with ratpool as a control were positive at an earlier stage (3 weeks; 4 passages) with a titre of 80.

3.5.2. Confirmation of BDV infectivity by cell titration

A cell ELISA was carried out with plasma from patient and controls to detect BDV antigen expressing cells (figure 3.24). A series of dilutions (log_{10} steps) of plasma were tested on the infected and control cells. Only the positive control cells and the cells infected with patient (Pte) stained positive. These results also showed that one infectious unit produced 20 – 60 antigen carrying cells in about 5 days (figure 3.24) and this was expressed as focus forming units per ml (ffu/ml) in table 3.7.

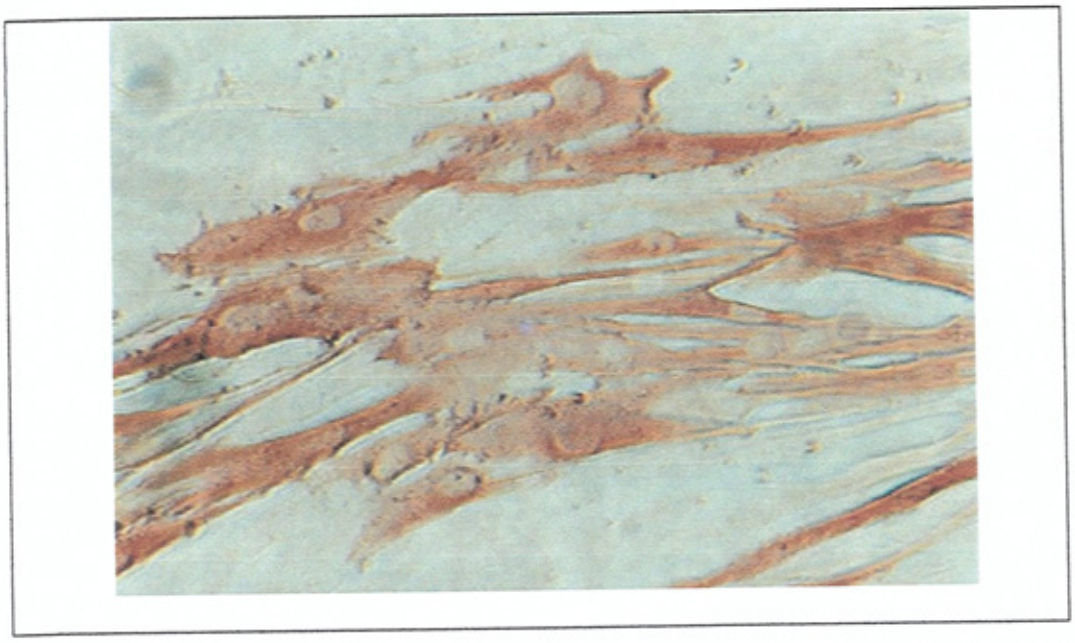

Figure 3.24: Detection of BDV antigen in the YRS cells with plasma of patient. Cells were probed with a mouse polyclonal serum to BDV and stained with substrate/stain mix (5 mg 3-amino-9-ethyl-carbazole (SIGMA), 5 ml Dimethyl Sulfoxide (Merck), Acetate buffer (242 g Tris base (2 M) + 57.1 ml Acetic acid + 100 ml of 0.5 M EDTA; see sub-section 2.5.3.2).

The number of virus positive cells (cells stained red) in each sample was counted and the final dilution of each sample containing positive cells is shown in table 3.7. The total number of virus infected cells and the Focus Forming Units (ffu) are also shown in table 3.7.

Specimen	Number of positive cells at final dilution	End-point titre	Virus containing cell/ml	FFU/ml*
Ratpool	20	10^{-3}	2.0×10^{4}	1×10^{5}
Sample 1 (Pte)	16	10^{-6}	1.6×10^{7}	8×10^{7}
Sample 2 (C1)	0	N/A	0	0
Sample 3 (C2)	0	N/A	0	0
YRS/TLp90/56	16	10^{-3}	8.0×10^{4}	4×10^{5}
OL/TLp55	25	10^{-3}	4.0×10^{3}	2×10^{4}
OL	0	N/A	0	0

Table 3.7: Number of viral infected cells in test samples tested and controls. 200 µl was dispensed into each well. The number of virus containing cells was calculated by multiplying the number of positive cells by 5 and by the dilution factor to give the ffu/ml.

This data demonstrate that the positive cells had considerable high titres of BDV. The negative OL cell control and hospital control (C1) as well as the blood donor controls (C2) were negative for BDV. Figure 3.25 reflects the dynamics of the viral load in the cells over the time period of this experiment.

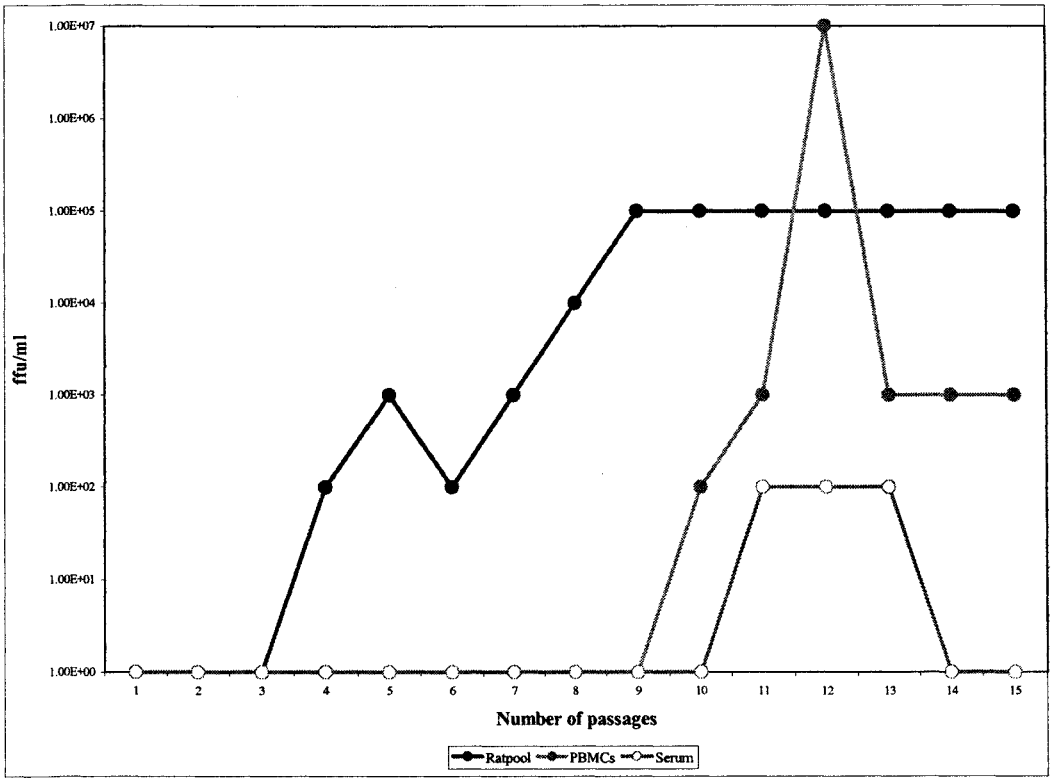

Figure 3.25: BDV virus specific CF-antigen in OL cell cultures. The lines represent the variation in BDV virus specific CF-antigen in OL cell cultures. Ratpool in the cell culture was used as the positive control and cultivated along with negative control and patient containing cells. PBMC and serum were from the Pte. Negative controls are not shown.

BDV was first detected in the OL cells cultured with rat pool 17 days after infection and titres of BDV increased over subsequent passages reaching a maximum titre of 4 x 10^5 ffu/ml 26 days post infection. After this the titre remained about the same. BDV was first detected in OL cells co-cultured with PBMC from Pte after 10 passages and the titre increased in a similar fashion as the BDV-positive OL cells, whilst cells co-cultured with plasma from Pte were positive for only three passages and then it tested negative.

3.5.3. Confirmation of infectivity by Western Blot (WB) Analysis

The WB analysis was used to identify specific BDV antigen in the test sample. Diluted serum/cell samples were treated with the detergent sodium dodecyl sulphate (SDS) and resolved by polyacrilamide gel electrophoresis. After blotting the cell suspensions onto nitrocellulose filter (figure 3.26). BDV proteins were identified by alkaline phosphatase-anti-rabbit IgG and Fast red/napthol-phosphate. Plasma from the BDV-ELISA-positive patient was assayed in parallel with the positive control serum. In both cases two bands were observed, at 38/40 kDa and a 24 kDa proteins indicated by closed arrow and open arrow respectively in figure 3.26. The negative control was also assayed in parallel and no bands were present (figure 3.26, lane 1).

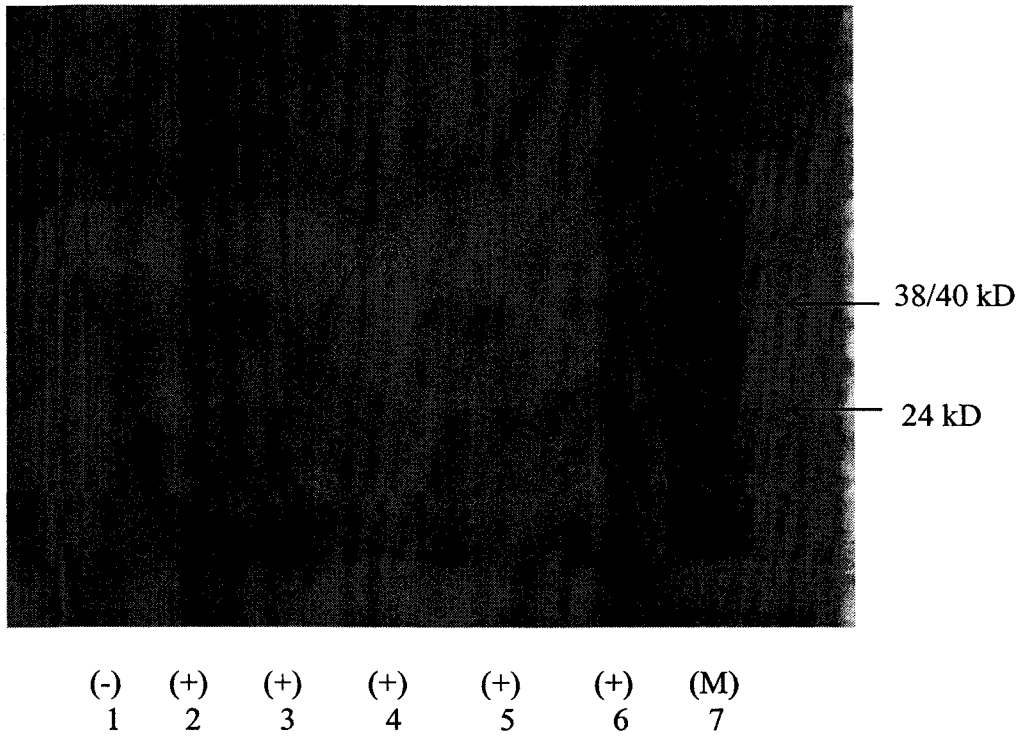

Figure 3.26: Western blot of BDV c-antigen from the MDD patient and the two controls (positive and negative) detected with BDV rabbit serum. 1 = Negative control; 2 and 3 = patient OL/Plasma; 4 and 5 = patient OL/PBMC; 6 = Positive control; 7= molecular weight marker (M); (-) = negative; (+) = positive. Bands for BDV proteins of 38/40 and 24 kD are present and shown with black arrow.

The samples in lanes 2, 3, 4, 5 and 6 which tested positive for BDV were all those samples that tested positive by other methods. Thus, the data presented here shows that OL cells were infected with BDV after incubation with Pte PBMC and plasma by all of the serological assays tested.

3.5.4. Confirmation of infectivity by molecular analysis (RT-nested-PCR)

Virus replication was also determined at every passage by RT-nested PCR on RNA extracted from the cells as described in table 3.8 and the results are presented for each passage with the exception of passage 4 to 11 where the results were always the same.

	P1	P2	P3	P4-P11	P12	P13	P14
Ratpool	-	-	+	+	+	+	+
Pte PBMCs	-	--	-	-	+	+	+
Pte Plasma	-	-	-	-	+	+	-
C1 PBMCs	-	-	-	-	-	-	-
C1 Plasma	-	-	-	-	-	-	-
C2 PBMCs	-	-	-	-	-	-	-
C2 Plasma	-	-	-	-	-	-	-
OL cells	-	-	-	-	-	-	-

Table 3.8: RT-nested-PCR confirming the presence of BDV RNA. P = passage; P4 - P11 = passage 4 to passage 11; Pte = patient; C1 = a member of the hospital control group; C2 = a member of the blood donor group; ratpool used as the positive control and OL cells used as the negative control. + = BDV RNA positive; - = BDV RNA negative.

During the 2 month period of the experiment OL cells co-cultured with ratpool and the patient plasma and PBMC were the only samples that tested positive for BDV-RNA.

An illustration of an RT-nested-PCR for the patient sample is shown in figure 3.27 along with the negative controls.

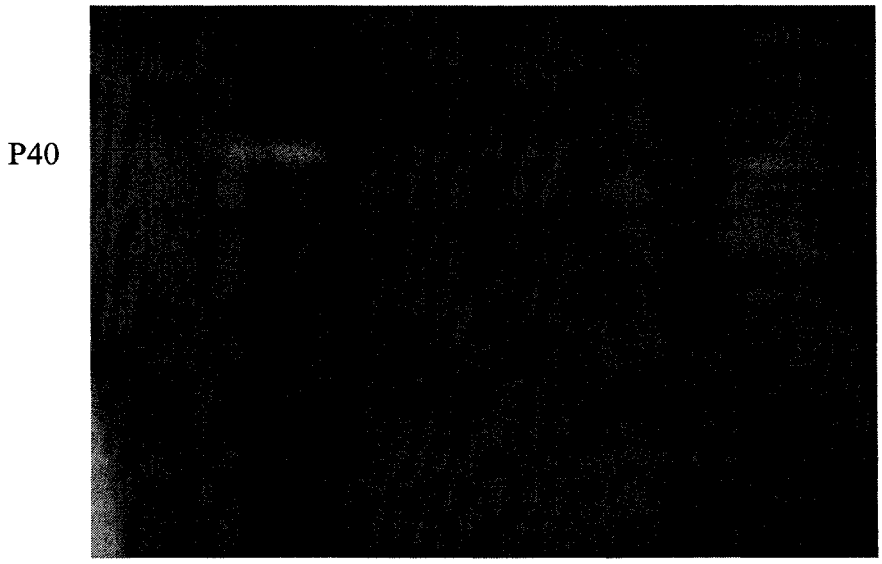

Figure 3.27: Detection of BDV RNA (p40) from patient (Pte). Disrupted cells were subjected to RT nested PCR. PCR products were resolved by agarose gel electrophoresis and visualised by staining with ethidium bromide M = marker; C1 = a member of the hospital control group; C2 = a member of the blood donor group; OL cells used as the negative control. (-) = negative; (+) = positive.

3.5.5. Summary of BDV isolation data

A range of tests were carried out to show BDV infectivity to OL cells with patient PBMC and plasma. All the tests carried out (morphology, IFA, RT-nested-PCR, Western blotting and cell ELISA) showed that the OL cells had been successfully infected with BDV (table 3.9).

Method	Patient (Pte)	Hospital control (C1)	Blood donor (C2)
Morphology	+	-	-
IFA	+	-	-
RT-nested-PCR	+	-	-
Western blot	+	-	-
c-ELISA (titration)	+	-	-

Table 3.9: Summary of test results for confirmation of BDV infectivity employed to samples from a patient, hospital control and blood donor in OL cells. Positive result (+) and negative result (-).

Thus this data shows that BDV can be isolated from both human PBMC and human serum and that it confirms that BDV is present in the population tested.

CHAPTER 4:

4. DISCUSSION

4.1. BDV seroprevalence in humans and horses

Borna disease virus (BDV) infects a wide variety of vertebrates and the similarity between behavioural syndromes and outcome in animals to that observed in humans, with a number of mental disorders (Ludwig & Bode, 2000) has led investigators to ask whether there is an association between BDV infection and major psychiatric diseases. A number of studies using serological and molecular evidence have shown an increased BDV prevalence in psychiatric patients as compared to healthy subjects (Lipkin *et al.,* 1995; de la Torre & Oldstone, 1996). This study is the first into BDV seroprevalence in the UK (on a cohort of 525 human samples and 272 horse samples). Both serological (triplet ELISA, IFA) and molecular diagnostic (RT-nested-PCR) methods were used in this study.

The key triplet Enzyme immuno assays (triplet ELISA) was used to screen for anti-BDV p40 and p24 IgG, p40 and p24 antigen, and circulating immune complexes (CIC) in the plasma (pAg; p40/p24 heteromers included). These three assays were performed on the same solid phase support and with the same volume per well, same buffers and same initial coating, as previously described by Bode and co-workers (2001), to try to ensure that the results were comparable to those of Bode.
The first assay on the triplet ELISA to consider is the antigen assay. By this test approximately 1/7 of the mood disorder patients tested positive (~15 %) and similarly about 1/5 individuals in the hospital control group were positive (19 %).

Interestingly only 1/15 of blood donors (9 %) tested positive for BDV antigen. Comparing these findings to the other markers tested for (antibody and CIC) on the triplet ELISA, it seems that the presence of antigen was consistently lower than the others possibly indicating an acute and productive but transient phase of infection. Other studies (Bode *et al.,* 2001) have shown that the severity of symptoms appears to correlate with the concentration and duration of antigenemia although this type of data was not available for this study.

Contrary to the results for humans, antigen detection in horses was consistently higher than the other two markers with about 13 % of horses positive for BDV antigen (table 3.3). It has been suggested that unlike humans a minority of BDV-infected horses can cope with high levels of antigenemia and these animals may remain healthy for several months (Ludwig & Bode, 2003), thus most horses with symptoms of Borna Disease present with strong antigenemia (Bode *et al.,* 2001). In this study there were no details of the horses and no follow ups were carried out thus whether these high levels of antigenemia render these horses at increased risk of disease is something beyond this study although it would be interesting to follow such a group of horses for a long period in the future.

The second assay was the antibody assay. Contrary to the antigen assay which shows active infection the results of this test could not be taken as an independent measure of infection as the presence of antibodies in a person's blood merely shows that they may have experienced an infection at some time in the past. In spite of this serology

based on the presence of anti-BDV antibodies is still widely used throughout the world. In this study antibodies were detected in about 29 % of patients, 28 % of hospital controls and 17 % blood donors. Thus there was a significantly higher seroprevalence as measured by anti-BDV antibody in the patient and hospital control population as compared to the blood donors ($p < 0.01$).

In the horses, anti-BDV IgG were not detected in animals from the Newcastle region which contrasts with horses from the national surveillance which had a considerable high seroprevalence rate (32 %). There is no obvious explanation for this but taking the groups together, about 12 % of horses were positive by the antibody assay.

The last assay on the triplet ELISA measured circulating immune complexes (CIC). This is a marker of BDV infection that few investigators have used for the detection of BDV infection. Bode and co-workers (2001) were the first to demonstrate the presence of circulatory immune complexes (CIC) in both animals and human and suggested that the presence of these free plasma complexes made from BDV antigen p40 and p24 and antibodies, may explain the disparity of results from laboratories. They stated that the prevalence of BDV-CIC indicated an infection rate of close to 100 % in acutely depressed patients (major depression or bipolar disorder), and up to 30 % in the healthy population. Thus, in previous human studies CIC seems to be present at a higher prevalence than both antigen and antibody. However, in this study the results for CIC were exactly the same as those ones found for the antibody test (table 3.1). If this test had not been used a lower number of individuals would be

reported positive here as the presence of antigen is critical in the diagnosis of disease. The quantitative analysis of CIC levels can allow a prognostic estimate of people with mood disorders, but, the significance of the presence of CIC remains controversial due to the present lack of turnover rate of BDV CICs. Contrary to the results for humans the percentage of positive horses by the CIC assay was lower than both the antigen and antibody assays although this was not significant with about 11 % of horses being positive for CIC. Unsurprisingly, no horses from the Newcastle region tested positive for the CIC test and it may be these horses were in a stage of acute infection and they had not yet developed IgG to BDV (Bode *et al.,* 1995) as CIC can only be only formed if antigens in the blood stream combine with circulating antibodies to produce the immune complexes (CICs). Thus we may assume that these animals were in a very early stage of acute infection, thus no antibodies are present and therefore CIC can not be made.

The average optical densities were higher for both the antigen and CIC in group 1 and 2 as compared to group 3 (figure 3.3). Interpreting this data is somewhat difficult as the OD is not strictly a measure of antigen and CIC concentration and there was no formal assessment on the "healthy" status of the control population but it suggests that there is more antigen and CIC in these groups. In the case of hospital control it has been reported that close contact with infected persons increases the risk of BDV infection (Bode & Ludwig, 2003). Blood donors are screened for a range of infections including hepatitis, syphilis, HIV etc (Schmunis *et al.,* 1998), but they are not submitted to a psychiatric assessment thus we cannot relate this data to clinical

status. A follow up of the severity of depression and the level of CIC and antigenemia was not made and thus cannot be further discussed but it would be interesting to look this in the future.

In summary a positive result for the triplet ELISA test was based on the presence of at least two markers for each sample. The technique detected native BDV antigens, anti-BDV antibodies or CIC to BDV p40 (viral nucleoprotein) and p24 (viral phosphoprotein) in sera. This method identified a total of 29 % of patients with mood disorders, 28% controls from a psychiatric hospital and 17 % of normal blood donors positive for BDV. There was no significant difference in seroprevalence of the patient group and the hospital workers control group ($\chi^2 = 0.02$ p=0.885) but the seroprevalence of BDV was significantly higher in the psychiatric patients and hospital workers as compared to blood donors ($\chi^2 = 14.92$ p=0.001). This data therefore shows that BDV infection and/or exposure is present in the UK with an increased prevalence in psychiatric patients complementing other studies showing that BDV is not limited to certain geographic areas in the world. The data presented here for blood donors is in contrast to another study in the UK, where no evidence of BDV infection in blood donors could be detected and the authors suggested that BDV in the UK population is not widespread (Davidson *et al,*. 2004). This conclusion was based on only one method (RT-PCR of plasma), even through it is well established that there is no standard method available for BDV detection. Further evidence to support this current study was presented by Chalmers *et al* (2005), who examined 50 published documents involving BDV and humans from

year 2000 - 2005 and came to the conclusion that humans were exposed to BDV and suggested that further epidemiological studies were necessary to establish associations with disease (Chalmers *et al.,* 2005). Nevertheless, the current study is presented with the limitation that samples have been obtained from only one area of the county and it should be noted that this may introduce bias in terms of UK representation. Thus, as Newcastle was the only area where samples were collected for this study the seroprevalence of BDV should be tested in other areas of the country.

It was also interesting to note that hospital workers exposed to psychiatric patients had similar high levels of BDV seroprevalence to those patients. This suggests that these individuals are exposed to high levels of BDV and may catch the virus at work, suggesting that BDV could have a human-to-human transmission route. This contrasts with a recent report from the UK, where investigators agree that although the healthy UK farming population may be exposed to BDV there is no association between BDV exposure and morbidity in occupational groups (Thomas *et al.,* 2005). However, this was a cohort study directed at healthy individuals and not a case-control study which allows comparison of differences between groups.

In support of the present study a similar study, involving schizophrenic patients, close family members of those psychiatric patients and mental health workers in direct contact with patients was conducted in Taiwan (Chen *et al.,* 1999a; Chen *at al.,* 1999b). This study showed that schizophrenic patients, mental health workers

and close relatives of schizophrenic patients had significantly higher levels of BDV RNA and serum antibodies than normal controls (14 %, 15 %, 12 % and 1.4 % respectively). There are no UK figures on the relative number of BDV infection markers to psychiatric diseases and psychiatric health workers but this data suggests that it would be important to study this group of individuals to determine if people who are exposed to the virus or who come in close contact with psychiatric patients are at greater risk of developing psychiatric diseases themselves.

The animals in this study had a lower prevalence rate of BDV infection (13 %) than humans and when compared to the reports from around the globe this number is lower than Turkey (25 %) although higher than USA (2.7 %). Thus, BDV infection in horses in the UK seems to be in the range of other countries throughout the world (Yilmaz *et al.,* 2002; Kao *et al.,* 1993).

Horse samples were from across the country and the results show that there was a wide variation in seropositivity between areas (table 3.3). Areas like Gloucester, Powys and Sussex had higher proportions of BDV seropositivity (5/7, 1/1 and 1/1 respectively). On the other hand Berkshire and Newcastle had lower proportions of BDV seropisitivity (1/21 and 4/78 respectively). There were also areas from which samples were all negative and these include Cheshire, Cornwall, Denbigh, Dorset, Essex, Hampshire, Hants, Lincs., N. Yorks., Norfolk, Oxfordshire, Shrops., Lancs., W. Midland, W. Sussex and Wilts although in the majority of cases only a small number were tested. It would be ideal to conduct further studies with representative

numbers of animals and clinical data from each area of UK to build a clearer picture on the seroprevalence of BDV in each region. Thus, whether one area truly had a higher seroprevalence rate is hard to tell because the number of samples was not the same for all regions e.g. Powys and Sussex only had one sample. In spite of this, the general point is that BDV has now been detected in horses in the UK and this is similar to other areas including most of the central European Countries (i.e. Germany, Switzerland, and Austria), America, Asia, Australia, and Africa (Bahmani *et al.,* 1996; Hagiwara *et al.,* 1997; Kao *et al.,* 1993; Malkinson *et al.,* 1995; Lundgren *et al.,* 1995; Hatalski *et al.,* 1997). Thus a detailed study of the prevalence of BDV in horses throughout the UK may be necessary and due to the link between BDV and human infection it would be prudent to study BDV infection in individuals who work in stables e.g. veterinarians exposed to horses.

The other technique used for serological diagnosis of BDV in this study was the Immunofluorescence assay (IFA). In this case, the fluorescent dyes fluorescein and rhodamine detected IgG to BDV in patients and horses by localised fluorescence in BDV infected cells. In a range of animals, antibodies have been found to be directed to the soluble antigen complex p40 and p24 (Furrer *et al.,* 2001) and it is also known that these antigens are actively transported from the cytoplasm to the nucleus where they accumulate (Ludwig *et al.,* 1985). Hence the IFA technique was used in this study to detect the presence of anti-BDV antibodies in human and animal sera. Internal controls in the form of the monoclonal antibodies (Kfu2 and W1HO) which detect the 40/38 kD and 24kD proteins respectively were also included. As with any

other diagnostic method there are limitations to its use but results from this part of the study increased our overall confidence in the results as a whole.

Positive horse sera normally present nuclear fluorescence although some also presented with cytoplasmic fluorescence as a result of the double-labelling of cells with test sera and the monoclonal antibodies (Kfu2 and WH8). In 27 horses (~10 % of positive) the staining pattern of horse serum matched that observed with the monoclonal antibodies (figure 3.14). The sera had an antibody titre of 40 which is in contrast to experimentally infected rabbit serum, which has a much higher titre of 360.

In the human study about 33 % of patients and 28 % of the hospital control group were positive by IFA whilst only 9 % of the blood donors were positive ($\chi^2 = 38.79$ $p<0.001$).

Comparing the results obtained by IFA and triplet ELISA the IFA seems to be a little more sensitive for the patient group as it detected about 3 % more cases although this was not significant ($\chi^2=0.22$ $p=0.638$). In contrast significantly less blood donors (Group 3) were positive for BDV by IFA (9 %) than by the triplet ELISAs (17 %) ($\chi^2=10.53$ $p=0.001$) whilst there was exactly the same number of positives detected in the hospital control group by both methods (table 3.1). A puzzling observation within this group was that although both methods had similar overall results the same individuals did not test positive or negative by both methods

(appendixes II) and similar differences in the reactivity of test serum have previously been reported by Kao *et al.,* (1993). This calls into question the specificity of both tests as genetic variability, environmental or immunological factors may interfere with the detection as suggested by other investigators (Muller-Doblies *et al.,* 2004, Bode *et al.,* 2001; Kolodziejek *et al.,* 2005; Horimoto *et al.,* 1997; Kao *et al.,* 1993; de la Torre, 2002; Bode & Ludwig, 2003).

IFA primarily recogned antibodies to structural viral proteins whereas the ELISA detected antibodies to native proteins (Bode & Ludwig, 2003). However, IFA had a major advantage over the triplet ELISA as its specificity was due to the incorporation of BDV-specific monoclonals in the IFA with a double-staining protocol. Conversely, the triplet ELISA has the advantage of speed, ease of performance, robustness and cost.

The results of this study are similar to other serological studies which consistently show an increase in BDV seroprevalence in neuropsychiatric patients with values in the regions of 10 to 50 % (Bode, 1995; Richt *et al.,* 1997b; Gonzalez-Dunia *et al.,* 1997; Lipkin *et al.,* 1997) as compared to healthy controls with much lower values between 0 to 25 % (Bode *et al.,* 1995; Kishi *et al.,* 1995a; Nakaya *et al.,* 1996; Sauder *et al.,* 1996).

The severity of depression was not measured in these patients and this may be important when testing for BDV in the human population. Some studies have shown

that those patients with Borna antibodies typically exhibit more pronounced symptoms of mental disorder than patients without antibodies (Carbone, 2001). Thus it may be that in the future a follow up study of the severity of psychiatric disorder and seroprevalence should be carried out as the absence of a viral marker at times may not exclude the possibility of infection as a case of seroconversion in psychiatric patients with BDV infection has been reported (Hallensleben *et al.*, 1998). Thus patients might first show no evidence of Borna infection but on subsequent testing, as depression manifests itself Borna antibodies may appear (Sauder *et al.*, 1996).

In general the results from the horse samples originating from Newcastle (regional) were consistently lower than the ones from the national surveillance. The results obtained from the IFA and ELISA were markedly different with the Newcastle group testing positive by IFA in 9 % of cases and only 5 % of cases by ELISA. The surveillance group showed an opposite pattern with fewer positive cases being detected by IFA (~10 %) than by ELISA (~13 %).

4.2. Molecular detection of BDV

Moving from the serological data to molecular data, RT-PCR assays have been used extensively to detect BDV RNA in both animals and humans. BDV specific RNA has also been shown in peripheral blood mononuclear cells of patients with psychiatric disease (Hagiwara *et al.*, 1996; Kishi *et al.*, 1995b; Kuratsune *et al.*, 1996). In order to verify whether viral nucleic acid was present in the blood, sera, and cells of the peripheral blood of human and horses in this study a reverse transcriptase reaction followed by nested-PCR amplifications were carried out in presence of specific primers. In the majority of cases, due to low level of virus material in the samples, the visible band of ~449 bp was only present on the gel, after nested PCR. This band corresponded to that fragment detected in the persistently infected YRS/TL cell line.

In general BDV-specific RNA was detected in 13 of 95 (14 %) patients with mood disorders (group 1). BDV specific RNA was also detected in 4 out of 32 (13 %) "healthy" volunteers from the hospital group 2 and in 25 (6 %) blood donors. Thus significantly more people in the psychiatric group and hospital worker group were positive for BDV RNA ($\chi^2 = 6.783$ $p= 0.034$) as compared to blood donors.

The prevalence rate by RT-PCR in sera of horses from both national and regional areas was 8 %. Previous analysis of the results by serology and results by RT-PCR

showed that Gloucester and Leicester had similar numbers of positivity by both methods (5 out of 7; 74.4 % and 1 out of 3; 33.3 % respectively). In contrast there were Republic of Ireland and Suffolk, which had lower infectivity rates by RT-PCR (9 out of 49; 18.4 % and 1 out 76; 1.3 % respectively) than by serology (17 out of 49; 34.7 % and 5 out 76; 6.6 % respectively).

Thus the data shows that some humans and horses are BDV positive although these results do show that testing for BDV RNA and anti-BDV IgG does not always give the same BDV prevalence. Subjects with positive nested PCR were not all positive for the triplet ELISA or IFA and vice versa. However, this data is similar to others where related inconsistent results have been demonstrated, in that BDV RNA and anti-BDV IgG may not be detected in the same subjects (Staeheli *et al.,* 2000; Carbone, 2001). It has also been reported that at times virus may or may not be present in individuals who test positive by serological assays as a positive serological result can indicate cure or "clearance" of the virus or a latent or persistent infection (Carbone, 2001; Bode, 1995). As we could not tell whether samples were from acute-phase or convalescent-phase we do not know how the detection of anti-BDV IgG relates to the presence or clearance of BDV and thus the detection rates with different diagnostic methods.

Other points to consider when evaluating this study is that only serum samples were tested and that the primers used in this study only detect p40. The majority of studies with higher levels of detection used primers against both p24 and p40 (Stoyloff *et al.,*

1997) and this may also account for the mismatch of results between the serological and the molecular methods as in the study antibodies to both p40 and p24 were used. When p24 and p40 BDV RNA and/or anti-BDV p24 or p40 antibodies were tested from human sera only rarely was there evidence of both BDV proteins in the same sample (Bode, 1995; Bode *et al.,* 1996; Kishi *et al.,* 1995a; Ogino *et al.,* 1998) therefore, the sensitivity of the test increases when looking for both antigen. Carbone (1997) also tried to clarify this inconsistency by taking into account the immunological factors involved in disease and stated that there may be the following three states in a host: "BDV is cleared from human tissues through an immune response (e.g., anti-BDV antibody positive and BDV antigen and RNA negative), BDV may be persistently replicating in the human host with a non-neutralizing immune response (e.g., anti-BDV antibody positive and BDV RNA positive) and finally BDV may be replicating in the human host without a measurable immune response (e.g., anti-BDV antibody negative and BDV RNA positive)".

Other causes for the low detection rates in this study may be due to the primers which were designed from animal virus sequences (strain V) and these may not be the same as human BDV. Another factor that could account for the lower sensitivity would be the presence of inhibitors (e.g. haemoglobin) in the sample, which could result in false-negative results or the low level of viral RNA in samples due to repeated freeze-thawing (Sierra-Honigmann, *et al.,* 1993, Sierra-Honigmann, *et al.,* 1995).

The use of a number of positive and negative controls showed the specificity of the RT-PCR results (BDV-infected and uninfected cells). Nevertheless the controls are possibly not the best as they are generally from cell lines that do not correspond to the samples (e.g., Young rabbit spleen cells or OL culture cells used as controls for human blood cell samples) giving a mismatch of sample and control cells in which cellular mRNA may vary.

A number of groups would not recommend the generation of PCR products of considerably more than 300 bp for diagnostic purposes (Zimmermann *et al.,* 1994; Richt *et al.,* 1997a; Dieffenbach *et al.,* 1995). In this study a nested PCR with 2 different sets of primers and 2 amplification reactions was used and this showed increased sensitivity, reliability and reproducibility over other studies. This assay was similar to that previously reported by Ludwig & Bode (1997) although some changes were made including the additional RNA isolation methods. After collection of blood in EDTA, RNA was isolated from whole blood, plasma or PBMCs using specific Qiagen kits. These kits are designed for each specific biological product, and have the advantage of having specifically designed columns which prevent contamination of the sample by substances such as phenol/chloroform which in turn may inhibit enzyme activities (Weitjens *et al.,* 1996). Moreover, Qiagen one step PCR seems to have advantages over other kits as it provides a master mix that prevents contamination and amplification of the synthesised cDNA without any loss of templates. One cannot ignore the fact that the appropriate conditions to carry out any RT-PCR are fundamental and so the work was performed under strict conditions

with different cabinets for each step of preparation, starting from separation of RNA, preparation of the reaction buffers, amplification and product analysis. Reagents were screened for contamination using negative reagent controls and RT-dependent amplification controls at regular intervals.

It is possible that more positive results would have been detected if other BDV proteins were investigated (Vahlenkamp *et al.,* 2000) but for this study it was decided to concentrate on the p40 coding region due to the restrictions of time and cost as it was planned to test all the samples by both serological and molecular methods. The decision to opt for the p40 coding region was mainly based on observations (Banerjee *et al.,* 1991; Conzelmann, 1998) that BDV, as other negative stranded RNA viruses, follows a pattern of replication and BDV p40 mRNA is among the most abundant viral transcripts present in infected cells and tissues (Planz *et al.,* 1999).

The final part of the molecular analysis of this study looked at sequence conservation between known tissue culture isolates and RT-PCR products derived from cells and sera of patients and controls that in turn were compared with the standard database with BLAST technology. Sequence analysis of the short nested PCR-products of 441 bp, and/or 449 bp verified the specificity of the PCR-products. Sequence divergences were of 0.1 to 5 % when compared to the reference strains V and He-80. The sequence analysis was limited to divergences with the two most common strains because other investigations (i.e. sequence confirmation) were not feasible.

Similar to other studies, the analysis of blood and sera from horses and humans gave inconsistent results. Firstly individuals which had either BDV-specific antibodies, antigen or CIC (BDV triplet markers) in the absence of viral RNA were detected. There were a number of samples that were positive for viral RNA in the absence of BDV-specific serological markers and some samples were positive for both BDV-specific triplet markers and viral RNA. These three groups have been seen in many studies in natural BDV infection but the phenomenon is not clearly understood (Kishi *et al.,* 1995b; Bahmani *et al.,* 1996; Sauder *et al.,* 1996 and Berg *et al.,* 1999). The presence of native protein, route of transmission, the biological sample analysed, specificity of monoclonals, primers, immune status and viral titre are some of the factors involved that may result in these discrepancies between the results of the different methods.

Analysing the results obtained from all of these techniques (triplet ELISA, IFA and RT-PCR) it was clear that results with different methods do not match. Some samples that tested positive for ELISA did not give the same results for one or more of the further tests (IFA, RT-PCR). The same was true for any test in the reverse order. WB analyses, which detected the binding of human antibody bound to BDV antigen, may have been more reliable and specific than IFA for confirmation of BDV serology, as it could be evaluated qualitatively by showing which virus antigen was recognised by the serum sample. Conversely, the technique had a major disadvantage of being time-consuming and expensive and as a result was only used on this study

for the isolated samples. Moreover, the ELISA method is reported to have higher sensitivity when compared to IFA method, due to the existence of cell-specific auto-antibodies, variability of reader interpretation and a lack of sensitivity for detecting low anti-BDV titres by IFA testing (Yamaguchi *et al.,* 1999; Hatalski *et al.,* 1997). RT-PCR was found by other investigators (Staeheli *et al.,* 2000) to be more sensitive than ELISA, being able to detect the presence of virus in latent phase of the infection, but this study only uses primers directed to detect the p40 proteins, which could account for the discrepancy of results.

From this discussion it is clear that there is a degree of controversy about the relevance of BDV detection by three methods due to inconsistencies between laboratories and techniques. For example two RT-PCR methods with the same QIAGEN kit were tested. Both amplified a different partial sequence of BDV under the same conditions and not surprisingly, the results showed that one method (Bode) was more sensitive than the other (Schwarz) although the second was much simpler to run. It must be remembered that nested PCR has other advantages over the second method such as the hot start that rendered its high specificity due to the combination of enzyme with the unique QIAGEN PCR buffer.

4.3.Assessment of assays

Independent assessment of assay variability was performed and 3 laboratories participated in a 2-day analysis of 5 and 10 blinded plasma samples for quality control in relation to IFA and RT-PCR. For the ELISA test only inter-laboratory variation in the absence of target values for the samples were analysed. Arbitrary reference values were used for all subsequent samples. The mean inter-laboratory variation in each method group ranged from 3% to 13%. The mean intra-laboratory variation was 2.6 % for plasma samples. Further, to ensure that testing results were as accurate as possible, considerable amount of time, effort, and money was made and those pre tested samples were used to calibrate and control testing.

The blinding of data sets also implied that a number was allocated to all samples and details of each one of them was only accessed at the analysis stage.

The mean positive value of 1.13 (95% CI 1.11 – 1.149) found in consecutive analysis of 5 positive samples, allowed the determination of the upper limit detection of positivity. Resetting the data to a lower detection limit would have skewed the data to the right as it would mean that more positive results would be found and therefore more than 5 % of false positive would have been reported. Taking group 2 (human) as an example and re-setting the data to 0.1 as the definite positive value, we would have got 2 more positive tests for the Ag detection, 8 more positive results for the anti-IgG test and 2 more positive results for the CIC test. Thus, the actual

seroprevalence of 28% would have been changed to 37%. However, it does not bring the results into a better line with the determinations as, even with this forged number, it did not change the fact that there would be less positive results for Ag and CIC as compared to the anti-IgG. Thus, the resetting of the data would not have had a significant implication on the results found for all groups tested.

4.4. Prevalence of BDV in humans and horses

All of the factors previously mentioned may have an impact on the prevalence of BDV detection, but they can not change the fact that the prevalence rate of BDV infection was always higher in humans with psychiatric disorders than healthy individuals which is similar to other studies (Bode et al., 1995; Bode et al., 1992; Kishi *et al.*, 1995 and Waltrip *et al.*, 1995). It also does not change the fact that BDV was detected at considerable levels in horses. Thus we can conclude from this section that BDV is present in both humans and horses in the United Kingdom. It was also found at a higher prevalence in individuals with psychiatric disorders as compared to normal controls and interestingly individuals who work with or in the vicinity of individuals with psychiatric disorders also have a significantly higher BDV prevalence. This finding adds and supports to other studies that human-to-human transmission may occur from psychiatric patients to family members and mental health workers in close contact with said patients (Chen *et al.*, 1999a, 1999b).

Definitive confirmation of BDV infection came with the isolation of BDV from peripheral blood cells of a patient diagnosed both with major depressive disorder and BDV and this will be discussed in section 4.4.

4.5. Evaluation of BDV infection in mood disorder patients and controls

BDV is a neurotropic virus that infects a range of animals and humans (Dietrich *et al.,* 1998). The virus seems to be present both in the general population and at higher levels in psychiatric patients (Fukuda *et al.,* 2001). This study determines the prevalence of BDV infection in individuals suffering from a range of mood disorders and controls included individuals working in direct contact with psychiatric patients and normal blood donors.

The prevalence of seropositive individuals reported worldwide range from 0 to 30% in the general "healthy" population, whilst seroprevalence in mood disorder patients generally range from about 1 % to 45 %. Examples include those studies by Rott *et al,* (1985), which showed a higher prevalence of anti-BDV antibodies (1.6 %) in patients with mood disorders than healthy controls (0 %) by IFA (Rott *et al.,* 1985). Waltrip *et al.* (1995) reported a significantly higher prevalence of anti-BDV antibodies in patients with schizophrenia (13.3 %) than in controls (0 %) again using

a WB technique. When looking at specific countries the majority of data on BDV infection in psychiatric patients, including Bipolar Patients (BP) are from Europe, America, and Japan with a range of prevalence rates from 25 to 45 % (Nakaya *et al.,* 1996; Bode *et al.,* 1995; Kishi *et al.,* 1995a).

The results of this study were broadly similar to others with patients having an infection prevalence rate of about 29 %. There was no significant difference in the prevalence of BDV in any of the patient groups except BPII patients who had a higher prevalence of BDV seropositivity. 6 out of 32 (19 %) hospital controls presented with high values of antigenemia and CICs and this rate seems to be very high when compared to other studies (Bode *et al.,* 2001). The difference in seropositivity between the hospital control group and the blood donors control group is striking and it raises the possibility that there was cross infection of the hospital control from the psychiatric patients, as they have increased levels of exposure to BDV. The selection of blood donors as a control group may have introduced some bias as it may not represent the general population (Lonnitto *et al.,* 2002), but as far as this study is concerned, this was an acceptable control group and has been used by several other studies on BDV seroprevalence (Terayama *et al.,* 2003; Takahashi *et al.,* 1997; Iwata *et al.,* 1998). It does assume that blood donors are healthy members of the community with a wide range of age and including both sexes. However, any further follow-up studies should be conducted with a properly chosen control group population with matching age, sex, and profession and all control members should undergo a psychiatric evaluation.

The data showing that BDV is present in the population and that higher levels of infection may occur in psychiatric patients with mood disorders and hospital workers, in psychiatric hospitals, is a potentially important observation for the public health point of view and further studies confirming these findings are urgently needed.

The higher prevalence rate of BDV infection in patients is also consistent with the hypothesis that BDV plays a role in the pathogenesis of neuropsychiatric disorders (Yamaguchi *et al.,* 1999). With regard to the lack of apparent symptoms in some individuals who are BDV positive, it was suggested that after infection BDV typically undergoes a period of little or no activity and this can last for several years thus potentially a person may be infected but never exhibit symptoms (Staeheli *et al.,* 2000). Occasionally, however Borna will begin a period of activity in which it speeds up its reproduction rate and more and more of the virus is released. The effect of this is a blocking of the signalling patterns between the central nervous system and the brain and the one vivid consequence of this signal blocking may be depression (Carbone *et al.,* 1989). The severity of an infection and its symptoms are now thought to be related to the particulars of an individual case such as age and immune status (Buchsbaum & Rieder, 1979). For example the age and sex of a population are critical when interpreting data on BDV prevalence as there are a number of studies that show a higher seroprevalence in the youngest quartile of patients (aged between 18 and 30 years) as compared with controls (Hallensleben *et al.,* 1998; Sauder *et al.,*

1996). This once again suggests a role of BDV infection in the pathogenesis of psychiatric disorders as psychiatric disorders frequently begin in younger persons (Rybakowski *et al.*, 2001).

In this study seroprevalence of BDV infection was compared between sexes and between different age groups (figure 3.6 and 3.7). Analysis showed that there was no significant difference in the rate of infection between males and females (χ^2= 0.04 p=0.836) when the data was pooled from all sets. Comparing the seroprevalence between sexes in the groups significantly more women in the hospital control group were seropositive than men (χ^2= 4.153 p=0.042) whilst there was no significant differences in the mood disorder group and blood donor controls (χ^2= 0.166 p=0.684; χ^2= 0.579 p=0.447 respectively). Overall this data suggests that BDV infection is not gender dependent as there was only a relatively small sample set in group 2, and this is consistent with the results reported by other researchers (Bechter *et al.*, 1992; Fu *et al.*, 1998).

With reference to age and BDV sero-positive status the dynamics were different in mood disorder patients and "healthy" subjects. The majority of mood disorder patients and blood donors that tested positive by the triplet ELISA test were in the age range 40 to 49 (39 % and 23 % respectively) whilst in the hospital control group the highest seroprevalence was in the age range 50 to 59 (56 %). Individuals younger than 30 or more than 60 years old were the least likely to be seropositive in the three groups (between 0 and 9 %; figure 3.6). Thus in general there was a steady increase

in number of positive individuals up to the age of 59 followed by a marked decrease in seroprevalence. This may suggest that BDV is age dependent and that after a prolonged period of infection certain individuals can clear this infection and they then become sero-negative and this observation has been made in other studies (Richt *et al.*, 1997b; Staeheli *et al.*, 2000).

4.6. Evaluation of BDV infection in horses

Samples from 274 horses were collected of which 196 had been collected during a routine national health surveillance scheme and were kindly provided by the Veterinary Laboratories Agency. These had been partially characterised by age (24 out of the 196) and geographic location. The second batch consisted of 78 samples generously provided by a veterinary clinic in the Northern region of England (Newcastle) and these were also partially classified by age. Results of the serological analysis, based on the triplet ELISA showed that 14 % of the horses were seropositive. Positivity was based on a sample testing positive for at least two of the markers tested. Of the 38 horses that tested positive for at least two markers 28 were positive by all three tests. This study was similar to other studies on BDV infection in horses where between 6.1 % and 29.5 % healthy horses have been shown to be

positive (Nakamura *et al.,* 1995; Berg *et al.,* 1999; Ludwig *et al.,* 1973; Kao *et al.,* 1993).

Again, it is hard to tell if the differences in seroprevalence are due to the methods of detection used in each study or if they really reflect the true actual state of infection within each country although it has been suggested that the penetration of BDV in horses may not be homogeneous throughout the world (Khan *et al.,* 2000). In this study it was noted that infection rates were dramatically different across the country although in the majority of cases only small numbers of horses were tested. A further large scale study of horses would be useful with age and clinical data included to define the positive rate of BDV in the UK.

4.7. Are we all infected by the virus?

Increasingly reports indicate that BD occurs world-wide (Germany, Switzerland, Austria, USA, Germany, the Netherlands, Poland, Israel, Iran, North Africa, Japan and now UK). Considerable epidemiological studies using both serological and molecular based approaches are highly indicative of BDV infection in humans (de la Torre *et al.,* 1996a; Chen *et al.,* 1999b; Amsterdam *et al.,* 1992) and suggest that BDV may be implicated in the pathogenesis of a number of psychiatric disorders e.g.

major depression, organic mood disorder, and obsessive-compulsive disorder. Yet, until recently BDV has not been considered a risk to the human populations. Further, clinically diagnosed neurologic disturbance and BDV reactive antibodies have been reported in specific occupational groups (e.g. farm workers; Hatalski *et al.,* 1997).

Studies enumerate that about one third of obsessive-compulsive disorder and schizophrenic patients show BDV antibodies (BDV was detected at higher frequencies in neuropsychiatric patients (30-66.7 %) (Kishi *et al.,* 1995a; Bode *et al.,* 1995) and in patients with chronic fatigue syndrome (24 %) (Nakaya *et al,* 1996) than in healthy controls (0-11 %) (Rott *et al.,* 1985; Igata-Yi *et al.,* 1996). The demonstration of such antibodies dates back to 1986 (Rott & Becht, 1995; Amsterdam *et al.,* 1985). In 1993/5 and 1999, Bode, Ferszt and their respective groups (Ferszt *et al.,* 1999; Dietrich *et al.,* 1998; Bode *et al.,* 2001) demonstrated the presence of BDV antigen and RNA in the peripheral blood mononuclear cells (PBMCs) of patients with major depression, panic disorder, obsessive compulsive disorder and organic affective disorder. In recent studies in Germany, BDV isolates from mood disorder patients were injected in animals and this lead to infection.

Generally patients infected by the virus present with anti-BDV antibodies, circulating immune-complexes (CIC) and antigens. A previous study from Germany suggested that CIC and antigen are present in higher rates in bipolar patients, unipolar depressed patients and schizophrenic compared to healthy controls (Bode *et al.,* 2001, Ferzt *et al.,* 1999) and that their frequency correlated with the severity of

depression and Bode (2001) suggested that BDV may have an etiological role in some psychiatric disorders.

Presently, there is little data from the UK analysing the presence and prevalence of BDV in persons with mood disorders. However, there is evidence from other countries such as Germany, USA, and Japan suggesting such a link. Viral infection rates may differ between countries, but taking a global view it should be noted that more and more countries are being added to the list of ones where BDV infection has been linked to psychiatric diseases.

The results observed in the present study do not contradict other studies; in fact it is supporting evidence, that BDV is present in the studied population with similar results as other groups when using similar detection methods.

4.8. Implications of the findings for public health

Borna Disease Virus is the causative agent of Borna Disease (BD), a disease responsible for the meningo- encephalomyelitis in animals and associated conditions such as unipolar affective disorders, panic disorder, chronic fatigue syndrome and some personality disorders (Saunder *et al.,* 1996; Nowotny & Windhaber, 1997; Kitani *et al.,* 1996). There are many studies showing an association between BDV

infection and psychiatric disorders suggesting that BDV infection may contribute to the onset of psychiatric symptoms (Bechter *et al.*, 1992; Rybakowski *et al.*, 2001). Thus in many studies BDV have been reported in healthy individuals although the risk of developing psychiatric diseases after infection is unknown.

CHAPTER 5:

5. CONCLUSION

5.1. General conclusion

Borna disease virus (BDV) is a neurotropic pathogen that infects a wide variety of vertebrates. The similarity in sequence between animal and human BDV isolates (de la Torre *et al.,* 1996a) and experimental data showing a broad species range for this agent suggest that animal-to-human transmission is a distinct possibility. Humans present with symptoms and disorders similar to those of BDV-infected animals such as paresis, weakness of legs, excitability and/or depression (Bechter *et al.,* 1992; Richt *et al.,* 1997b). Thus, there have been a number of investigations on the possible association between BDV infection and major psychiatric disorders in humans both serological and molecular evidence shows that individuals with a number of psychiatric disorders have higher levels of BDV infection markers (anti-BDV antibodies, BDV antigen and BDV CIC) as compared to healthy subjects (Lipkin *et al.,* 1995; de la Torre & Oldstone, 1996).

Normally, viral spread depends very much on the infection route and the development or the reactivity of the immune system at the time of infection (de la Torre *et al.,* 1996; Chiu *et al.,* 1999). In addition it has been reported that horizontal transmission of BDV is possible (Bechter *et al.,* 1992; Richt *et al.,* 1997b; Hagiwara *et al.,* 2000). BDV disseminates intra-axonally in peripheral nerves (Carbone *et al.,* 1987; Morales *et al.,* 1988). In some reports BDV can be detected in peripheral nerve endings, but not in parenchymal cells of visceral organs, with the exception of

medullar cells of the adrenal gland (Narayan *et al.,* 1983b; Gosztonyi *et al.,* 1984; Carbone *et al.,* 1987), after intracerebral inoculation, although in some cases BDV has been detected in parenchymal cells of a range of organs and exocrine glands (Herzog *et al.,* 1984; Schamel *et al.,* 2001). This study has shown that BDV p40-RNA can be detected in both PBMCs and sera of horses and humans in the UK, although it may expressed to a higher extent in other parts of the body.

BDV is currently a subject of much investigation throughout the world to determine its prevalence and its effects on humans and animals. In this study BDV diagnosis was performed by either the demonstration of virus-specific antibodies, antigen, CIC in sera/plasma and RNA in PBMCs. Several investigators have applied the same or similar techniques in other areas of the world and throughout there is much controversy in the field of BDV research as to the relevance/sensitivity/specificity of these detection methods, a range of techniques were used in this study in order that these data could be related to the world literature.

Sera and blood samples from human and horses in the UK were analysed by triplet ELISA, IFA, WB and RT-PCR and infection rates in this study were similar to those seen in other studies (Igata-Yi *et al.*, 1996; Kishi *et al.*, 1995; Bode *et al.*, 2001).

This study also confirmed previous studies which showed inconsistencies in the detection of BDV RNA and anti-BDV antibodies from the same subject (Staeheli *et al.,* 2000) as some samples were positive by serological analysis and PCR and some

were only positive for one or the other. However, it is suggested that the range of tests used here for BDV detection give a reasonable estimate of thr proportion of individuals infected.

Overall the human results from this study show that there was a higher prevalence of BDV infection in the sample of psychiatric patients (29%) as compared to the blood donor controls (17%). It also shows that individuals that were in close contact with psychiatric patients, the hospital control group, had similar infection rate (28%) to the sample of the psychiatric patients and this was also significantly higher than the blood donor controls (17%).

This data therefore clearly shows that BDV, or an analogous virus, is present in the studied population. The question of whether BDV infections in humans contribute to human neuropsychiatric disorders however remains unsolved. The fact that BDV was also found in "healthy" individuals does not challenge this hypothesis since it is suggested that the natural infection can persist sub-clinically for many years and a yet unsolved process may trigger its clinical effects.

Apart from the small sample sizes, the major limitation to this study is the lack of clinical assessment in the control subjects as it was assumed that all individuals in these groups were healthy with no major clinical manifestation at the time of blood collection, although this was not possible to determine within the design of the current study.

The observation that the control group from the hospital had similar BDV prevalence rates to the mood disorders patients leads to the speculation that cross infection by direct contact between patients and health workers is possible. This is a quite alarming prospect if the link between BDV and psychiatric disease is finally fully established and further studies on this area should be carried out as soon as possible. One also has to ask whether BDV is a work-related disease or is it an emerging zoonosis. Another potential are for concern is the transmission of BDV during blood transfusion. There have been a number of cases in Australia whereby multi-transfused patients present with higher seroprevalence of BDV than the blood donors (personal communication, Dr Sandra Kamhieh). The implications of BDV infection in these multi-transfused subjects are unknown but it has been speculated that may play an influential role in the outcome of other conditions (i.e. aplastic anaemia, acute myelogenous leukaemia).

In relation to the UK horse population, this study shows that BDV is present in the UK with levels similar to those reported in Germany, Iran, USA, Sweden and Japan. The fact that there was no diagnostic assessment for the animals did not allow the comparison of prevalence of Borna disease in the UK with other countries. Nonetheless, the study permits the conclusion that BDV is present in the horse population in the UK.

It would be of great interest to analyse samples from a range of animal species in the UK to establish the true prevalence and incidence of BDV within animals in the UK. Further work should also be carried out in the United Kingdom to determine whether specific populations are at increased risk of BDV infection (psychiatric health workers, farmers) and may help determine possible routes of exposure.

Previous studies have shown that BDV can infect granulocytes and be detected in PBMCs. In this study PBMC from a chronically depressed patient with a major depressive disorder, who was consistently BDV positive with high levels of infectious markers, were used to infect a cell line with virus. These infected cells were passaged and a persistent infection was demonstrated in these cells. Further evidence to that fact that the cells were infected was the observation that the viral titre increased with time indicating that active synthesis of virus specific material must have taken place. This study therefore confirms the presence of BDV in PBMCs and further confirmed the results obtained by the serological and molecular methods in this patient.

Similarly to other persistent viral infections it is suggested that BDV is found at low concentrations in the blood as shown by the relatively long time it took to be detected in cell culture, but after reaching the CNS and initiating long-term chronic infection levels will increase and may cause slowly progressive neurological disorders which may be associated with diverse pathological manifestations.

This study has also shown that cells are susceptible to BDV infection and that BDV may be transmitted by direct contact (cell-cell) although whether BDV represents one of the environmental cofactors associated with neuropsychiatric disorders is still unknown and beyond the scope of this study.

In conclusion this study shows that:

1. BDV antigen, anti-BDV IgG and BDV RNA can be detected in both humans and horses.
2. There is a higher seroprevalence of BDV in these psychiatric patients and psychiatric health workers sampled/tested as compared to blood donor samples suggesting that human to human transmission occurs.
3. BDV can be isolated from PBMC of infected individual and cultured for long periods *in vitro*.

5.2. Future directions

In the past two decades BDV research has impacted on various fields, not only the biology of neurotropic RNA viruses but also neuropsychiatry. Although several discoveries regarding this unusual pathogen have been made some major issues concerning its biology and pathology remain to be resolved. Despite numerous reports of BDV infection in humans, problems including the range of seroprevalence detected between laboratories, low levels of antibody titres, and sequence similarities between human BDV isolates and horse-derived laboratory strains all raise fundamental questions as to whether BDV infects humans and whether it contributes to neuropsychiatric disorders. Although the broad host range of BDV suggests that humans are targets for infection, the sources and routes of human infection are unclear. Furthermore, the size of the population of animals affected by BDV outside the classical endemic areas is not yet known. Detailed epidemiological studies using reliable diagnostic techniques should be performed on domestic animals around the world.

A more thorough understanding of the molecular and cellular biology of BDV is needed to explain the neuropathogenesis of this virus. The functions of each viral protein, as well as the mechanism of persistent infection by the virus in the nucleus have not been fully determined. Determination of the regulation of viral gene

expression in the nucleus will further our understanding of the unique cell tropism of BDV in the CNS. The generation of infectious molecular clones of BDV using a reverse-genetic system would be a breakthrough approach in this field.

BDV is highly neurotropic, noncytolytic and induces long-lasting persistent infection both *in vivo* and *in vitro*. Since large amounts of viral proteins are detected in infected cells, determining the detailed effects of viral proteins on cellular functions will also provide insight into the mechanisms whereby CNS infection of BDV can result in neuro-developmental damage and neurobehavioral disorders in the host.

Ultimately further samples should be analysed alongside health questionnaires to examine in more detail the clinical significance of this potentially serious zoonosis.

Thus there is an urgent need for large scale epidemiological and pathological studies to rigorously evaluate the contribution of BDV to human psychiatric disorders and the potential role of infection of man by animals.

CHAPTER 6:

6. REFERENCES

6.1. REFERENCES

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APPENDICES

Reagents and Solutions

1. Enzyme immuno assays (ELISA)

➢ Sample dilution buffer: (PBS-T) pH 7.2 + 0.05 % Tween 20: (8 g NaCl; 0.2 g KCl; 0.2 g KH_2PO_4; 2.9 g $Na_2HPO_4.2H_2O$; 0.2 g NaN_3 dissolved in 1000 ml distilled H_2O)

➢ Conjugate buffer: TBS-T pH 8.0 (TRIS 0.02 M): (2.4 g TRIS; 8.0 g NaCl; 0.2 g KCL dissolved in 1000 ml distilled H_2O pH adjusted with HCL)

➢ Coating buffer (pH 7.6): 0.01 M tribasic sodium phosphate (Na3PO4)/ 0.25 M NaCl pH 7.6 (65 ml solution A (2.76 g of 0.02 M $NaH_2PO_4 \cdot H_20$ to 1000 ml distilled water); 435 ml solution B (3.361 g of 0.02 M $NaHPO_4 \cdot 2H_20$ to 1000 ml distilled water); 14.6 g 0.25 M NaCl adjusted to 1000 ml distilled water and stored at 4°C).

➢ Wash buffer: 0.9 % NaCL + 0.05 % Tween 20 + 0.02 % Na-Azide (2.5 g /ml Tween 20; 45 g NaCL; 1.0 g NaN_3 dissolved in 5000 ml distilled H_2O; Ultrawash Plus Dynatech Labs, Chantilly, VA, USA)

➢ Substrate buffer: Diethanolamine (2-(2-Hydroxyethylamino) ethanol) (Merck) buffer pH 9.8 (0.1 g $MgCl_2.6\ H_2O$; 0.2 g NaN_3 in 843 ml distilled water and 60 ml 1 N HCl)

➢ Stop solution: 3 M NaOH (120 g NaOH added to 1000 ml distilled water)

➢ 10 mM sodium phosphate + 250 mM sodium chloride, pH 7.6

➢ P-nitrophenylphosphate (pNPP) 1mg ml^{-1} (SIGMA N-2765; 20 mg/tablet)

➢ 1 M Diethanolamine buffer (pH 9.8) + 0.5 mM Magnesium Chloride

➢ 3 M Sodium Hydroxide

2. Immunofluorescence assay

➢ Phosphate Buffered Saline (PBS): pH 7.4

8 g NaCL; 0.2 g KCl; 0.2 g KH_2PO_4; 1.44 g $Na_2HPO_4.2H_2O$ dissolved in 1000 ml distilled H_2O

➢ 0.2 M EDTA:

Ethylendiamintetraacetic acid (EDTA) 7.45 g dissolved in 100 ml distilled H_2O and adjusted to pH 7.4 with HCL.

➢ Trypsin solution

Trypsin 2.5 g was dissolved in 100 ml PBS and sterile filtered and stored at -20°C until use. For cell passage 1 % (v/v) 0.2 MEDTA was added. This solution was also obtained ready made 0.25 % Trypsin -EDTA (SIGMA: 100 ml; contains 2.5 g porcine trypsin and 0.2 g EDTA.4Na/L Hank's Balanced Salt Solution (HBSS); stored in aliquots of 25 ml at 20°C. Gibco RRL - life Technologies: Unit 100 ml)

➢ Penicillin/Streptomycin solution:

625 mg Benzylpenicillin and 700 mg streptomycinsulphate were dissolved in 100 ml distilled water. The solution was filtered and stored at -20°C until further use. This antibiotic was also obtained ready made (10 000 IU ml^{-1} - 10 000 $\mu g\ ml^{-1}$) from Gibco RRL - life Technologies

➢ Complete cell culture medium:

Dulbecco's modified eagle medium (DMEM: Gibco, without Sodium Pyruvate, with 4500 mg L^{-1} Glucose, with Pyridoxine HCL; 500 ml) sterile filtered, Penicillin/Strptomycin solution at a final working of 0.1 v/v and 5 % New-born Calf Serum (NCS: mycoplasma screened; Gibco RRL - life Technologies: Unit 100 ml)

3. Detection of BDV RNA in cells of the peripheral blood and plasma/serum by RT-PCR

- Buffer RLT (Lysis buffer containing guanidine isothiocyanate and 10 µl of b-Mercaptoethanol (b-ME) per 1 ml of Buffer RLT)
- Buffer RW1 (contains guanidine isothiocyanate and ethanol, Qiagen)
- Buffer RPE (elution buffer dissolved in 4 volumes of ethanol (96–100 %) Qiagen)
- 0.25 % trypsin- Ethylene diamine tetraacetic acid (EDTA)
- Phosphate Buffered Saline (PBS): pH 7.4

  8 g NaCl; 0.2 g KCl; 0.2 g KH_2PO_4; 1.44 g $Na_2HPO_4.2H_2O$ dissolved in 1000 ml distilled H_2O
- 5 x Qiagen OneStep RT-PCR Buffer (5 % concentrated. Contains Tris-HCl, KCl, $(NH_4)_2SO_4$, 12.5 mM $MgCl_{2)}$
- dNTP Mix; Qiagen (10 mM each of dATP, dCTP, dGTP, and dTTP; ultrapure quality)
- Qiagen OneStep RT-PCR Enzyme Mix (20 mM Tris-Cl, 100 mM KCl, 1 MM dithiothreitol (DTT), 0.1 mM EDTA, 0.5 % (v/v) Nonidet P-40, 0.5 % (v/v) Tween 20, 50 % glycerol (v/v), stabilizer; pH 9.0 (20°C))
- HotStarTaq DNA Polymerase (Qiagen; 5 U)
- RNase-free water; Qiagen (Ultrapure quality, PCR-grade)

- RNasin® Ribonuclease Inhibitor (Supplied in 20mM HEPES-KOH (pH 7.6), 50mM KCl, 8mM DTT, 50 % (v/v) glycerol Source: Human placenta (Promega UK Ltd~ Delta House ~ Chilworth Research Centre ~ Southampton SO16 7NS ~ United Kingdom)
- 2 mM dNTP Mix ((containing 2 mM of each dATP, dCTP, dGTP and dTTP an 2mM aqueous solutions (Helena Bioscience-MBI Fermentas Helena Bioscience-MBI Fermentas; Colima Avenue, Sunderland Enterprise Park, Sunderland, SR5 3XB)
- Storage Buffer: 10 mM TrisHCl, pH 7.4 - 1mM EDTA
- Blue/Orange 6X Loading Dye (G188A): (10 % Ficoll 400, 0,25 % xylene cyanol FF, 0,4 % orange G, 10mM Tris-HCl (pH7.5) and 50 mM EDTA)
- TE buffer:1 mL of 1 M Tris (pH 8.0)[10 mM): 200 µL of 0.5 M EDTA, disodium salt (1 mM) and 99 mL distilled water

4. Confirmation of BDV infectivity by means of focus assay

- Fixative solution (Formalin (10.8 ml of 37 % formaldehyde added to 100 ml PBS, pH 7.4)
- PBS-Triton-X 100 (Sigma)

- Reaction buffer, pH 5.0 (12 ml of 2.4 % (v/v) ethanoic acid added to 400 ml dH_2O titrated with 1 N NaOH pH 5.0 and 500 ml dH_2O, filter-sterilised and stored).
- Peroxidase-conjugated anti-mouse IgG(Fc)
- Substrate/stain mix (5 mg 3-amino-9-ethyl-carbazole (SIGMA), 5 ml Dimethyl Sulfoxide (Merck), Acetate buffer (242 g Tris base (2 M) + 57.1 ml Acetic acid + 100 ml 0.5 M EDTA. Make to 1 litre in distilled H_2O to pH 5.0), PBS-Triton-X 100 (Sigma)
- 30 % Hydrogen Peroxide (Merck).
- Virus suspension: 2.5 ml of DMEM containing 100 µl of the virus
- Tested sample suspension: 50 µl of OL, OL/TL, PBMC and plasma in 450 µl DMEM supplemented with 0.1 % Pen/Strep and 10 % FCS, adjusted to contain 4 x 10^5 cells per ml).
- Uninfected cell suspension: OL cells or YRS at 10^4 cells l^{-1} (10 ml DMEM to 1 plate of trypsinased cells)

5. BDV infectivity confirmation by Western Blotting (WB)

- Transfer Buffer

Tris 15.15 g, Glycerine 72.00 g, Methanol 1000 ml, added 5000 ml distilled H_2O

- Blocking Buffer

  10 % (v/v) Roti-Block 10x in PBS + 0.05 % Tween-20

- Phosphate Buffered Saline (PBS): pH 7.4

  8 g NaCl; 0.2 g KCl; 0.2 g KH_2PO_4; 1.44 g $Na_2HPO_4.2H_2O$ dissolved in 1000 ml distilled H_2O

- Acrylamide - bis acrylamide mix : 40 % of 37.5:1 ratio
- Main Gel Buffer (1.5 M Tris - HCl pH 8.8)
- 10 % Ammonium persulphate
- TEMED
- Stacking Gel Buffer (0.5 M Tris - HCl pH 6.8)
- 5 x Running Buffer: 45 g Tris Base; 216 g Glycine; 15 g SDS; volume adjusted to 3 litres with distilled water
- Blotting Buffer: 15.15 g Tris base; 72.10 g glycerine ; 1l methanol; volume adjusted to 5 litres with distilled water
- Gel Preservative Solution: 100 ml Acetic acid; 100 ml glycerol; volume adjusted to 1 litre with distilled water.

- Blot Wash Buffer: 150 mM NaCl; 100 mM Maleic acid pH to 7.5 with NaOH; 0.3 % Tween-20
- Radish peroxidase-conjugate goat anti-human IgG (Sigma)
- Alkaline Phosphatase Buffer: 100 mM Diethanolamine pH 10.0; ~5 mM $MgCl_2$
- 75 mg/ml nitro blue tetrazolium chloride (NBT, disolved in 70 % dimethyl formamide)
- 25 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt, (BCIP, dissolved in 100 % dimethyl formamide)
- Main gel: 0.5 ml SDS; 0.5 ml Ammonium Persulphate; 40 µl TEMED
- 10 % Acrylamide gel: 12.5 ml acrylamide /bis 40 % mix; 16.7 ml main Gel Buffer; 20.8 ml distilled water
- Stacking Gel (5 %): 3.125 ml Acrylamide / bis 40 % mix; 3.5 ml Stacking Gel Buffer; 18.375 ml distilled water. After de gas add 0.25 ml SDS; 0.25 ml Ammonium Persulphate and 20 µl TEMED.

RESULTS OF HUMAN SAMPLES (GROUP 1)

Patients with major depressive disorder (MDD)

N.	Age	Sex	Occupation	Ag	1.	2.	Ab	1.	2.	CIC	1.	2.	IFA	PCR
1	59	M	retired prison officer	-	20	13	+	192	282	-	34	20	+	+
2	60	M	unemployed	-	17	′7	-	61	63	-	64	83	-	-
3	47	F	housewife	-	19	18	?	114	206	-	122	118	-	-
4	63	F	labourer ret	-	5	5	?	119	136	?	104	108	+	-
5	38	M	nurse RGN	-	24	53	++	317	227	+	230	154	+	-
6	46	F	teacher ret	-	18	3	-	33	72	-	142	152	-	-
7	49	M	ex-clerk	-	4	6	-	64	101	+	195	103	-	-
8	58	M	ex-driver	-	58	53	+	246	184	++	301	301	+	-
9	54	F	unemployed	-	80	115	-	35	67	-	40	24	-	-
10	38	F	housewife	+	164	110	+	186	172	+	241	163	+	-
11	33	M	incapacity	++++	1057	623	+++	827	865	++++	1129	750	+	+
12	43	F	executive	-	88	38	-	96	112	-	28	19	-	-
13	59	M	incapacity	?	122	73	-	37	52	-	59	63	-	-
14	28	F	secretary	-	88	45	-	44	113	-	42	34	-	-
15	58	F	housewife	-	91	50	+	204	194	+	161	143	-	-
16	39	F	housewife	-	31	67	+	230	263	++	353	308	+	-
17	56	M	engineer	-	40	48	-	50	99	-	50	53	-	-
18	52	F	clerk	-	38	35	-	71	95	-	91	115	-	+
19	47	M	ex- miner	-	98	79	?	117	152	-	119	114	-	-
20	39	M	farm work	+	289	161	++	460	369	++	451	80	+	-
21	54	F	teaching	-	87	43	-	63	98	+	150	446	-	-

N.	Age	Sex	Occupation	Ag	1.	2.	Ab	1.	2.	CIC	1.	2.	IFA	PCR
22	57	F	engineer	-	74	49	?	109	150	-	67	301	-	-
23	59	M	crane driver	-	76	40	?	110	161	-	80	158	-	-
24		M	GP	?	124	74	-	64	44	-	87	50	-	-
25	52	M	shepherd	-	42	25	+	121	231	++	341	426	+	-
26	30	F	unemployed	+	191	91	+++	652	903	++	545	518	+	-
27	51	F	housewife	-	94	40	+	176	178	+	278	263	+	+
28	47	F	housewife	-	43	26	-	61	76	+	178	127	-	-
29	48	F	housewife	-	105	44	-	96	156	-	109	129	-	-
30	56	M	miner	?	79	99	-	47	56	-	78	90	+	-
31	47	F	housewife	-	26	26	-	67	38	-	80	76	-	-
32	53	M	incapacity	-	25	32	-	63	98	-	55	31	-	-
33	40	M	landscape gardener	+	204	134	+++	645	558	+++	782	327	+	+
34		M		?	81	61	-	38	83	?	106	113	-	-
35	30	M	invalidity	-	80	115	-	35	67	-	40	24	-	-

Table II-a.1: Patients with major depressive disorder (MDD). Ag = antigen test results; 1. = OD1; 2. = OD2. Note that OD values are presented without the decimal places. OD value less than 0.079 were negative; OD values between 0.079 and 0.149 were all retested.

Patients with bipolar I rapid cycling (BPIRC)

N.	Age	Sex	Occupation	Ag	1.	2.	Ab	1.	2.	CIC	1.	2.	IFA	PCR
36	31	F	administration	-	50	16	+	224	176	+	153	194	+	-
37	43	F	nurse	+	141	92	+++	614	463	+	223	193	+	+
38	50	M	unemployed	-	53	23	-	103	207	-	29	18	-	-
39	42	M	dentist	+	167	88	++	466	594	++	416	199	+	+
40	39	F	ex-care assistant	-	61	72	-	49	70	?	102	78	-	-
41	56	M	salesman	-	78	79	-	83	71	?	110	231	-	-
42	54	M	invalidity	-	85	41	-	94	83	-	33	9	-	-
43	54	F	ex-teacher	-	54	84	-	28	107	-	46	39	-	-
44	47	M	unemployed	-	37	26	-	70	94	-	99	120	-	-
45	41	F	teacher	+	276	249	+++	704	733	+++	881	365	+	+
46	48	M	retired teacher	?	125	80	-	58	57	?	102	129	-	-
47	48	M	unemployed	-	45	41	-	89	58	+	182	191	+	-
48	39	M	driver	-	58	27	-	74	102	+	226	392	+	-
49	53	F	vol. work	-	39	20	-	60	85	+	166	345	-	-
50	59	M	ret. engineer	-	74	41	-	76	84	-	32	39	-	-
51	55	F	housewife	-	70	49	-	67	79	+	159	165	-	-
52	39	F	driver	-	22	24	-	47	50	-	67	49	-	-
53	59	M	engineer	-	54	32	-	37	47	-	136	105	+	-
54	41	F	dentist	+	191	131	++	532	409	++	507	207	-	-

Table II-a.2: Patients with bipolar I rapid cycling (BPIRC). Ag = antigen test results; 1. = OD1; 2. = OD2. Note that OD values are presented without the decimal places. OD value less than 0.079 were negative; OD values between 0.079 and 0.149 were all retested.

Patients with Bipolar I not-rapid cycling (BPInotRC)

N.	Age	Sex	Occupation	Ag	1.	2.	Ab	1.	2.	CIC	1.	2.	IFA	PCR
55	56	M	Taxi driver	-	29	7	-	4	31	-	132	154	-	-
56	52	M	mathematician	-	96	89	+++	773	316	-	29	14	-	-
57	54	F	voluntary work	-	10	6	-	50	116	?	102	181	-	-
58	56	M	invalidity	-	18	13	-	81	107	+	263	156	-	-
59	35	M	IT trainer	-	48	33	?	119	116	-	67	100	-	-
60	53	M	lecturer	-	8	0	+	190	225	+	215	199	+	-
61	50	M	toolmaker	-	78	58	+	231	266	+	260	144	+	-
62	46	F	voluntary work community	-	22	21	-	48	121	-	49	22	+	-
63	34	F	worker	-	43	44	-	17	65	-	13	10	-	-
64	41	F	sewing machinist	-	50	72	-	47	76	-	90	45	-	-
65	48	F	unemployed	-	70	42	-	73	92	?	114	199	-	-
66	43	F	unemployed	?	134	73	+	181	210	-	35	55		-
67	43	M	ex-electrician	-	79	67	-	30	40	-	142	106	-	-
68	55	M	manager	-	73	38	-	99	87	-	130	101	-	-
69	28	F	student	-	87	47	-	69	128	?	122	155	-	-
70	52	F	embroider		88	45	-			-			-	-
71	44	F	occupational therapist	+++	625	362	++	445	380	++	463	242	+	+
72	55	M	incapacity	?	125	91	-	90	104	-	80	96	-	-
73	62	M	unemployed	-	40	36	-	49	79	-	86	101	-	-
74	43	M	carpet fitter	-	70	64	+	207	284	++	427	347	+	-
75	53	F	Shop assistant	-	89	40	+	164	195	-	52	25	-	-

N.	Age	Sex	Occupation	Ag	1.	2.	Ab	1.	2.	CIC	1.	2.	IFA	PCR
76	35	M	unemployed	-	75	50	+	260	350	++	398	432	-	-
77	48	F	cleaner	+	292	170	+	213	502	++	409	506	+	+
78	50	F	Shop owner	-	38	29	-	63	102	++	300	350	-	-
79	49	F	shop assistant	-	68	32	?	104	101	+	194	121	-	-
80	52	M	IT co-ordinator	-	50	23	-	95	90	-	15	12	-	-
81	47	M	dentist	-	22	45	-	69	124	-	96	135	-	-
82	58	F	unemployed	-	63	27	-	22	71	-	227	160	-	-
83	28	F	student	-	42	23	-	87	46	+	165	141	-	-
84	43	F	housewife											

Table II-a.3: Patients with Bipolar I not-rapid cycling (BPInotRC). Ag = antigen test results; 1. = OD1; 2. = OD2. Note that OD values are presented without the decimal places. OD value less than 0.079 were negative; OD values between 0.079 and 0.149 were all retested.

Patients with Bipolar II not-rapid cycling (BPIInotRC)

N.	Age	Sex	Occupation	Ag	1.	2.	Ab	1.	2.	CIC	1.	2.	IFA	PCR
85	42	F	carer	-	23	4	++	376	437	++	369	166	+	-
86	45	F	nun	-	77	79	+	164	142	+	265	214	+	-
87	25	M	student	+	277	185	+++	883	643	+++	827	327	+	+
88	37	M	unemployed	+	167	192	++	508	872	++	545	563	+	+
89	23	M	student	-	51	25	-	60	139	-	101	125	-	-

Table II-a.4: Patients with Bipolar II not-rapid cycling (BPIInotRC). Ag = antigen test results; 1. = OD1; 2. = OD2. Note that OD values are presented without the decimal places. OD value less than 0.079 were negative; OD values between 0.079 and 0.149 were all retested.

Patients with Bipolar II rapid cycling (BPIIRC)

N.	Age	Sex	Occupation	Ag	1.	2.	Ab	1.	2.	CIC	1.	2.	IFA	PCR
90	43	F	social worker	++	368	138	++	338	450	+	250	246	+	+
91	49	M	ex-teacher	-	21	5	-	45	72	-	67	63	-	-
92	61	F	ex-TV make-up artist	-	60	28	+	170	506	++	521	285	+	-
93	43	F	assistant manager	-	50	25	-	39	62	+	151	158	-	-
94	44	M	unemployed	-	35	19	-	96	109	-	122	113	-	-
95	34	F	lecturer	-	36	27	-	108	87	-	39	23	-	-

Table II-a.5: Patients with Bipolar II rapid cycling (BPIIRC). Ag = antigen test results; 1. = OD1; 2. = OD2. Note that OD values are presented without the decimal places. OD value less than 0.079 were negative; OD values between 0.079 and 0.149 were all retested.

RESULTS OF HUMAN SAMPLES (GROUP 2)

Hospital control

N.	Age	Sex	occupation	Ag	1.	2.	Ab	1.	2.	CIC	1.	2.	IFA	PCR
1	37		professor	-	121	80	-	54	52	-	60	137	-	-
2	28		nurse	-	23	21	-	47	33	-	77	98	-	-
3	38		nurse	-	77	69	?	102	52	-	43	68	-	-
4	43		nurse	-	69	82	-	99	115	-	69	102	-	-
5	50		nurse	-	75	80	?	107	90	-	49	61	-	-
6	56		ret'd admin	+	182	211	+	228	157	+	169	131	-	-
7	59		nun	-	98	79	-	29	17	(+)	138	156	-	-
8	37		nurse	+	201	144	+	252	149	+	278	262	+	-
9	38		nurse	-	193	139	++	538	252	+++	688	467	+	-
10	47		nurse	-	78	42	-	138	162	-	92	93	-	-
11	44		nurse	-	110	52	+	276	239	-	97	147	-	-
12	51		domestic	-	43	43	-	119	170	-	33	77	-	-
13	48		engineer	-	88	40	+	170	289	+	150	243	+	-
14	59		chaplain	-	84	40	+	184	416	?	109	47	-	-
15	57		lecturer (retired)	(+)	121	39	++	427	347	++	535	600	+	-
16	37		psychiatrist	-	30	28	++	327	173	+	178	214	+	-
17	50		support worker	++	542	389	++++	1252	669	+++	736	915	+	+
18	43		secretary	-	33	27	?	111	168	-	82	55	-	-
19	43		clinic admin	-	40	65	?	116	164	-	71	87	-	-
20	46		ASW	-	51	28	+	187	186	-	49	58	-	-

N.		Sex	occupation	Ag	1.	2.	Ab	1.	2.	CIC	1.	2.	IFA	PCR
21	50		psychiatrist	-	61	30	+	156	172	-	13	51	-	-
22	60		retired foreman electrical engineer shop	-	54	37	+	222	207	-	41	68	-	-
23	59		assistant	-	57	45	+	249	245	-	34	85	-	-
24	57		drug-rep.	-	106	73	-	27	14	-	96	177	-	-
25	46		ASW	-	21	21	-	157	106	-	99	133	-	-
26	49		nurse	-	7	13	+	276	103	-	55	124	-	-
27	50		social worker	-	20	10	?	103	100	+	185	355	+	+
28	51		ASW	++	576	444	++++	1245	845	++++	1553	1464	+	+
29	49		receptionist	-	85	30	+	239	183	++	396	352	+	-
30	24		secretary	-	33	18	-	64	78	-	82	127	-	-
31	52		childminder	++	598	156	++	564	328	++	551	415	+	+
32														

Table II-b.1: Ag = antigen test results; Ab = antibody test result. 1. = OD 1; 2. = OD 2. Note that OD values are presented without the decimal places. OD value less than 0.079 were negative; OD values between 0.079 and 0.149 were all retested.

RESULTS OF HUMAN SAMPLES (GROUP 3)

BLOOD DONORS

Number	Year born	Sex	AG	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
1	1947	f	-	20	22	-	46	19	-	-	-
2	1960	f	-	35	30	-	34	54	-	-	-
3	1947	m	-	19	13	-	9	3	-	-	-
4	1939	f	-	19	19	-	28	22	-	-	-
5	1951	m	-	19	26	-	43	38	-	-	-
6	1933	f	-	30	21	-	73	56	+	-	-
7	1935	f	++	353	176	++	471	259	+	+	+
8	1940	m	-	41	33	+	287	193	+	-	-
9	1946	m	-	24	17	-	13	36	-	-	-
10	1939	f	???	107	30	++	303	464	+	-	-
11	1977	m	++	360	241	++	326	397	+	+	+
12	1948	f	-	34	24	-	33	51	-	-	-
13	1952	f	-	17	8	-	3	39	-	-	-
14	1974	m	-	28	22	-	41	86	+	-	-
15	1953	f	-	32	17	+	172	125	-	-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
16	1968	f	++	359	218	+++	787	403	+	+	+
17	1948	m	-	51	29	-	68	201	+	-	-
18	1965	m	+++	849	620	+++	950	486	+	+	+
19	1953	m	-	26	4	-	51	47	+	-	-
20	1960	f	-	5	3	-	80	38	+	-	-
21	1940	f	-	20	22	-	71	52	+	+	-
22	1968	m	-	28	9	???	101	47	+	-	-
23	1956	m	-	39	20	+	210	188	+	-	-
24	1970	f	-	24	22	+	253	73	+	+	-
25	1959	m	+	196	98	+++	664	310	+	+	+
26	1954	f	-	82	45	+++	747	206	+	+	-
27	1969	m	-	7	11	(+)	138	108	-	-	-
28	1945	m	-	12	5	-	21	85	-	-	-
29	1961	m	-	16	14	+	151	89	-	-	-
30	1952	m	-	50	17	++	383	131	+	-	-
31	1936	f	-	17	7	-	68	50	-	-	-
32	1938	m	++	330	153	+++	865	494	+	+	+
33	1976	f	-	26	16	-	73	82	-	-	-
34	1940	m	-	7		-	92	76	-	-	-
35	1958	f	-	17	-2	-	71	40	-	-	-
36	1959	m	-	4	8	-	67	38	-	-	-

Appendix II-c:

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
37	1938	m	-	5	-1	-	72	35	-	-	-
38	1956	m	++	543	475	++++	1,115	943	+	+	+
39	1964	f	-	22	0	-	73	27	-	-	-
40	1944	m	-	14	-2	-	59	27	-	-	-
41	1948	f	-	8	1	-	58	79	-	-	-
42	1952	m	+	153	35	+++	608	246	+	+	+
43	1949	m	+++	639	633	+++	890	601	+	+	+
44	1944	f	-	21	14	(+)	133	67	-	-	-
45	1965	f	-	17	2	-	87	33	-	-	-
46	1942	f	-	25	-4	-	64	44	-	-	-
47	1965	m	+	271	196	++	503	646	+	+	-
48	1964	m	-	68	25	+	275	167	+	-	+
49	1944	m	-	28	8	-	92	82	-	-	-
50	1956	m	-	22	5	-	48	36	-	-	-
51	1956	f	-	16	5	-	53	60	-	-	-
52	1957	m	-	28	7	-	33	114	-	-	-
53	1959	m	-	14	6	-	72	55	+	-	-
54	1945	m	-	19	7	-	33	47	-	-	-
55	1958	m	-	40	17	-	72	51	-	-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
56	1938	f	-	10	-4	+	219	175	-	-	-
57	1946	m	++	546	381	+++	631	364	+	+	+
58	1944	m	-	2	3	-	66	27	-	-	-
59	1958	m	-	-4	-4	(+)	132	37	-	-	-
60	1954	m	-	9	-2	-	76	45	-	-	-
61	1950	f	-	31	-4	???	111	38	-	-	-
62	1953	f	+	267	118	+	283	258	+	-	+
63	1983	f	+	192	37	++	476	374	+	+	-
64	1944	f	-	4	6	-	9	26	+	-	-
65	1967	f	-	6	3	-	10	23	-	-	-
66	1939	f	-	18	10	-	28	58	+	-	-
67	1960	f	-	11	3	-	98	59	-	-	-
68	1937	m	-	30	32	-	80	153	-	-	-
69	1967	m	-	12	10	+	226	168	+	-	-
70	1949	m	-	41	20	???	110	80	+	-	-
71	1980	m	-	16	3	-	81	49	+	-	-
72	1949	f	-	37	14	-	98	95	+	-	-
73	1944	m	-	26	-8	(+)	122	46	+	-	-
74	1960	f	-	28	2	-	90	41	-	-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
75	1958	f	-	21	7	???	120	103	-	-	-
76	1952	f	-	28	13	-	80	64	+	-	-
77	1939	f	-	48	31	++	311	129	+	-	-
78	1933	m	-	-1	-3	-	26	38	-	-	-
79	1939	f	-	2	3	-	61	69	+	-	-
80	1942	m	(+)	138	67	+++	654	555	+	+	+
81	1977	f	-	15	0	-	28	67	-	-	-
82	1967	f	-	11	0	(+)	123	201	-	-	-
83	1964	f	-	28	-1	-	48	45	-	-	-
84	1971	f	+	226	148	+++	788	461	+	+	+
85	1951	m	-	4	0	-	72	88	-	-	-
86	1962	m	-	21	7	+	232	202	+	-	-
87	1941	m	-	25	15	-	89	80	-	-	-
88	1956	m	-	24	18	-	40	130	-	-	-
89	1956	m	-	84	112	+	152	79	-	-	-
90	1983	f	-	52	72	-	78	112	-	-	+

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
91	1946	m	-	75	53	-	63	71	-	-	-
92	1948	m	-	87	73	-	73	59	-	-	-
93	1953	m	+	154	82		89	117	+	-	-
94	1955	f	+++	964	795	++++	1,065	1.194	+	+	+
95	1968	m	-	87	61	-	45	76	-	-	-
96	1960	f	+	198	114	+	185	114	+	-	+
97	1949	m	-	95	65	???	108	81	-	-	+
98	1957	f	+	188	109	++	353	586	+	+	-
99	1946	f	-	90	92	-	75	85	-	-	-
100	1966	m	-	54	49	-	42	71	-	-	-
101	1967	f	-	55	44	-	33	48	-	-	-
102	1957	m	-	58	59	-	48	66	+	-	-
103	1937	f	-	60	81	-	20	39	-	-	-
104	1962	m	-	49	53	-	40	41	-	-	-
105	1956	m	???	117	89	+	279	502	+	+	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
106	1958	f	-	71	65	-	33	27	-	-	-
107	1977	m	-	70	68	-	63	143	-	-	-
108	1946	f	-	58	57	-	48	67	+	-	-
109	1954	f	-	60	77	-	11	41	-	-	-
110	1968	f	-	67	83	-	30	70	-	-	-
111	1962	m	-	15	7	-	97	77	-	-	-
112	1967	f	-	12	-2	-	42	67	-	-	-
113	1956	m	-	5	2	-	50	52	-	-	-
114	1957	f	-	15	9	-	88	71	-	-	-
115	1959	m	-	23	-12	+	217	128	+	-	-
116	1952	m	-	35	13	+	173	344	+	-	-
117	1959	m	-	18	3	-	55	27	-	-	-
118	1954	m	-	22	17	-	87	45	+	-	-
119	1963	f	++	435	125	+	271	152	+	+	+
120	1980	m	-	25	2	-	51	40	-	-	-
121	1973	f	-	22	18	-	51	47	+	-	-
122	1960	m	-	22	18	+	230	94	+	-	-
123	1949	f	-	3	10	-	63	25	-	-	-
124	1961	f	-	15	9	-	17	15	-	-	-
125	1975	m	-	31	23	(+)	143	52	+	-	-
126	1966	m	-	-15	-13	-	78	26	-	-	-
127	1956	f	-	16	17	-	21	25	-	-	-
128	1960	m	-	14	19	???	103	50	-	-	-
129	1977	f	-	8	11	(+)	149	71	-	-	-
130	1972	m	-	22	24	-	49	28		-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
131	1979	f	-	12	6	-	10	11	-	-	-
132	1958	f	-	8	12	-	22	15	-	-	-
133	1944	f	-	2	-9	-	44	43	-	-	-
134	1952	f	-	-12	-17	-	42	70	+	-	-
135	1962	f	-	10	-11	-	35	46	-	-	-
					-						-
136	1967	f	-	4		-4 -	71	32	-	-	
137	1955	f	-	-33	-40	-	31	32	-	-	-
138	1954	m	+	178	156	+	336	489	+	-	+
139	1961	m	-	-26	-18	-	93	25	+	-	-
140	1933	f	-	-18	-28	-	4	18	-	-	-
141	1958	f	-	-15	-32	-	23	10	-	-	-
142	1962	m	-	-10	-19	-	30	13	+	-	-
143	1951	m	-	-22	-20	-	15	30	-	-	-
144	1960	m	-	-16	-20	-	66	53	-	--	-
145	1951	f	-	-19	-20	-	87	63	+	-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
146	1979	f	-	17	15	+	208	289	+	-	-
147	1951	m	-	-19	-16	-	24	31	-	-	-
148	1954	f	-	36	15	(+)	126	51	+	-	-
149	1958	m	-	-6	-10	-	52	36	-	-	-
150	1964	m	-	-14	-18	-	32	13	-	-	-
151	1941	f	-	-31	-14	-	32	28	-	-	-
152	1941	m	-	-21	-26	-	19	14	-	-	-
153	1963	m	-	-16	-28	-	8	10	-	-	-
154	1971	f	-	-23	-21	-	15	12	-	-	-
155	1967	f	-	-1	4	-	43	40	-	-	-
156	1963	f	-	-4	0	-	29	10	-	-	--
157	1960	f	-	38	14	(+)	146	55	-	-	-
158	1959	f	-	40	18	-	42	42	-	-	-
159	1965	f	-	-29	3	-	30	8	-	-	-
160	1966	f	-	-1	15	-	86	25	-	-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
161	1939	f	-	-4	0	-	51	51	-	-	-
162	1962	f	+	215	229	++	467	456	+	+	-
163	1968	f	-	0	3	-	30	17	-	-	-
164	1968	f	-	5	37	-	9	23	-	-	-
165	1938	m	-	8	19	-	27	25	-	-	-
166	1972	m	-	12	-1	-	61	17	-	-	-
167	1947	m	-	218	73	???	110	39	+	-	-
168	1966	m	-	9	-1	(+)	136	43	+	-	-
169	1965	f	-	-6	-5	-	14	14	-	-	-
170	1934	f	-	-7	-12	-	6	19	-	-	-
171	1973	f	-	-2	6	-	10	21	-	-	-
172	1971	m	-	-5	68	-	12	4	-	-	-
173	1963	f	-	37	15	-	85	19	-	-	-
174	1953	m	(+)	142	48	(+)	126	39	+	-	-
175	1960	f	-	-6	-4	-	10	8	-	-	-

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
176	1959	f	-	8	2	-	13	17	-	-	-
177	1979	f	-	15	3	-	19	47	-	-	-
178	1971	f	-	-9	-12	-	11	69	-	-	-
179	1961	f	-	2	-7	-	35	35	-	-	-
180	1976	f	-	29	-2	+	189	87	+	-	-
181	1946	f	-	13	-3	-	62	56	+	-	-
182	1949	f	-	21	0	-	95	74	-	-	-
183	1973	f	-	4	-5	-	24	25	-	-	-
184	1955	m	-	6	-9	(+)	127	135	+	-	-
185	1977	m	-	-5	1	-	39	20	-	-	-
186	1960	f	-	-4	-12	-	37	35	-	-	-
187	1956	f	-	8	-3	-	21	33	-	-	-
188	1956	f	-	1	-5	-	61	27	-	-	-
189	1961	f	-	-9	-20	-	36	16	-	-	-
190	1963	f	-	-10	-14	-	27	18	-	-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
191	1946	m	-	-4	-5	-	18	26	-	-	-
192	1965	f	++	470	167	++	383	236	+	+	+
193	1946	f	-	11	75	-	7	4	-	-	-
194	1974	f	-	38	10	-	75	18	+	-	-
195	1950	m	-	-1	3	-	29	16	-	-	-
196	1960	f	-	-10	-9	-	22	21	-	-	-
197	1960	f	(+)	128	-9	-	5	4	-	-	-
198	1969	f	-	-6	1	-	34	32	-	-	-
199	1965	f	-	26	15	-	29	30	-	-	-
200	1953	m	-	11	4	-	48	72	+	-	-
201	1970	m	-	6	9	-	28	17	-	-	-
202	1965	m	-	13	-3	-	63	40	-	-	-
203	1954	m	-	-2	-6	-	22	27	-	-	-
204	1952	m	-	19	-3	-	84	78	+	--	-
205	1943	m	(+)	149	67	+	180	206	+	-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
206	1971	m	-	-4	-12	-	28	79	+	-	-
207	1950	m	-	-10	-10	-	10	81	+	-	-
208	1969	f	-	-8	-6	-	25	53	+	-	-
209	1940	m	-	-5	-12	-	38	51	+	-	-
210	1955	m	-	-26	-37	-	20	99	-	-	-
211	1960	f	-	-8	-8	-	9	100	-	-	-
212	1982	m	-	-7	-8	-	58	45	+	-	-
213	1949	m	-	-13	11	-	14	26	-	-	-
214	1978	m	-	2	-8	-	16	34	+	-	-
215	1970	f	-	0	-8	-	19	58	-	-	-
216	1980	f	-	-9	-18	-	43	84	-	-	-
217	1970	f	-	-13	-10	-	16	45	-	-	-
218	1963	m	-	-6	-6	-	19	27	-	-	-
219	1963	m	-	12	-5	-	22	112	+	-	-
220	1958	f	-	6	-4	-	59	50	+	-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
221	1972	f	-	27	20	???	118	109	+	-	-
222	1972	f	-	20	7	-	77	87		-	-
223	1965	m	???	114	27	+++	812	596	+	+	+
224	1934	m	-	26	14	+	160	124		-	-
225	1946	f	-	22	13	???	107	168	+	-	-
226	1962	m	-	71	17	+	298	116	+	+	-
227	1957	m	-	29	20	???	118	146		-	-
228	1938	m	-	27	22	-	9	64	+	-	-
229	1954	m	-	6	17	-	8	57		-	-
230	1953	f	-	24	5	-	90	88		-	-
231	1969	f	-	31	17	++	336	236	+	+	-
232	1980	f	-	30	-4	++	343	181	+	-	-
233	1976	f	-	42	11	(+)	127	139		-	-
234	1964	f	-	23	34	-	33	64		-	-
235	1961	m	-	18	-1	+	157	110		-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
236	1968	f	-	14	9	-	48	59	-	-	-
237	1978	f	-	31	14	-	88	88	-	-	-
238	1948	m	-	19	11	-	77	78	-	-	-
239	1966	f	-	20	8	++	388	199	-	-	-
240	1941	f	-	9	12	-	69	60	+	-	-
241	1941	f	-	4	-9	-	95	62	-	-	-
242	1951	f	-	1	45	-	84	57	-	-	-
243	1956	m	-	14	4	-	94	53	-	-	-
244	1949	f	-	10	-3	(+)	129	39	-	-	-
245	1943	m	-	14	-11	+	171	126	-	-	-
246	1966	m	-	19	-2	+	164	92	+	-	-
247	1951	m	-	17	-3	-	64	45	-	-	-
248	9164	f	-	4	-3	+	171	82	+	-	-
249	1950	f	-	14	10	-	47	53	-	-	-
250	1959	m	-	7	36	???	112	102	-	-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
251	1974	m	-	4	-10	-	62	57	-	--	-
252	1966	m	-	11	-9	-	60	86	-	-	-
253	1953	f	-	19	2	+	163	127	+	-	-
254	1950	f	-	24	17	-	90	102	+	-	-
255	1971	m	-	-10	-19	-	87	92	-	-	-
256	1960	f	-	-8	-17	-	92	86	-	-	-
257	1964	m	-	-6	-11	-	69	72	-	-	-
258	1959	f	-	-6	-11	(+)	139	101	-	-	-
259	1967	m	-	-20	-16	(+)	144	87	-	-	-
260	1979	m	-	52	11	++	313	209	+	+	-
261	1975	m	-	40	3	+	159	36	+	-	-
262	1959	m	+	288	55	++	330	174	+	+	+
263	1962	f	-	13	-7	-	68	42	-	-	-
264	1967	m	-	13	19	???	107	105	-	-	-
265	1965	f	-	21	5	-	74	362	-	-	-

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
266	1975	m	(+)	148	33	+	251	173	+	+	-
267	1937	m	-	16	5	-	52	83	-	-	-
268	1946	f	-	35	11	-	54	393	-	-	-
269	1961	f	-	19	6	+	250	658	+	-	-
270	1961	f	-	40	9	-	68	110	-	-	-
271	1952	f	-	52	27	-	55	119	-	-	-
272	1955	m	-	14	8	-	78	99	-	-	-
273	1950	f	(+)	137	42	++	565	347	+	+	+
274	1942	f	-	45	14	+	170	248	-	-	-
275	1957	f	-	26	14	++	344	262	+	+	-
276	1972	f	-	18	3	-	71	110	-	-	-
277	1961	f	-	11	-24	???	119	158	-	+	-
278	1963	m	+	256	18	+	159	274	+	+	-
279	1963	m	-	14	4	-	19	50	-	-	-
280	1944	f	-	-7	4	- ???	103	114	-	-	-

Human samples

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
281	1960	f	-	16	6	-	10	8	-	-	-
282	1947	f	-	18	8	(+)	133	115	-	-	-
283	1960	m	-	7	6	-	94	91	+	-	-
284	1968	f	-	11	2	???	104	4	-	-	-
285	1983	f	-	-9	8	-	73	88	+	-	-
286	1949	m	+	212	237	+++	723	505	+	+	-
287	1963	f	-	13	12	-	78	104	-	-	-
288	1957	f	-	13	11	(+)	124	115	-	-	-
289	1953	m	-	-2	12	-	98	91	-	-	-
290	1949	m	-	14	3	-	76	66	-	-	-
291	1938	m	-	25	8	+	178	158	-	-	-
292	1947	m	-	7	10	???	76	102	-	-	-
293	1954	m	-	11	8	+	107	116	-	-	-
294	1958	m	-	40	17	+++	184	234	-	-	-
295	1971	f	++	301	303	(+)	642	637	+	+	+

Number	Year born	Sex	Ag	OD1	OD2	CIC	OD1	OD2	Ab*	IFA	PCR
296	1968	f	-	19	14	++	127	172	-	+	-
297	1952	m	(+)	122	57	+	311	200	+	+	-
298	1980	f	-	16	17	++	214	131	-	-	-
299	1941	f	-	38	16	-	365	307	+	-	-
300	1955	f	-	17	14	-	95	99	-	-	-

Table II-c.1: General summary of results from blood donors. M= male; f = female; optical densities (OD) values presented without the decimal places; * OD values for the antibody assay are not presented. Results of 102 blood donors are not shown. Ag = antigen test; Ab = antibody test

Summary of results from Newcastle equine samples

Sample n.	Ag	Ab	CIC	IF	PCR
1	-	-	-	-	-
2	-	-	--	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
6	-	-	-	+	+
7	-	-	-	-	-
8	-	-	-	-	-
9	-	-	-	-	-
10	-	-	-	+	+
11	-	-	-	-	-
12	++	-	-	+	+
13	-	-	-	-	-
14	-	-	-	-	-
15	-	-	-	+	-
16	-	-	-	-	-
17	-	-	-	-	-
18	-	-	-	-	-
19	-	-	-	-	-
20	-	-	-	-	-
21	-	-	-	-	-
22	-	-	-	-	-
23	-	-	-	-	-
24	-	-	-	-	-
25	-	-	-	-	+
26	-	-	-	-	-
27	-	-	-	-	-
28	-	-	-	-	-
29	-	-	-	-	-
30	-	-	-	-	-
31	-	-	-	-	-
32	-	-	-	-	-
33	-	-	-	+	-
34	-	-	-	-	-
35	-	-	-	-	+
36	-	-	-	-	-
37	-	-	-	-	-
38	-	-	-	-	-
39	-	-	-	-	-

Sample n.	Ag	Ab	CIC	IF	PCR
40	-	-	-	-	-
41	-	-	-	-	-
42	-	-	-	-	-
43	-	-	-	-	-
44	-	-	-	-	-
45	-	-	-	-	-
46	-	-	-	-	-
47	-	-	-	-	-
48	-	-	-	-	-
49	-	-	-	-	-
50	+	-	-	-	-
51	-	-	-	-	-
52	-	-	-	-	-
54	-	-	-	-	-
55	-	-	-	-	-
56	-	-	-	-	-
57	-	-	-	-	-
58	-	-	-	-	-
59	-	-	-	-	-
60	-	-	-	-	-
61	-	-	-	-	-
62	-	-	-	-	-
63	-	-	-	-	-
64	+	-	-	+	+
65	-	-	-	-	-
66	-	-	-	-	-
67	-	-	-	-	-
68	-	-	-	-	-
69	-	-	-	-	-
70	-	-	-	-	-
71	-	-	-	-	-
72	-	-	-	-	-
73	-	-	-	-	-
74	-	-	-	-	-
75	+	-	-	+	-
76	-	-	-	-	-
77	-	-	-	-	-
78	-	-	-	-	-

Table III-a.1: All results presented as positive or negative. OD values are not shown. +=positive; -=negative. Ag = antigen; Ab = antibody.

General summary of national surveillance's horses

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
1	Berks	VLA00-0 45605		-	60	45	-	57	61	-	60	85	-	-
2	Suff	VLA00-0 45755		-	65	63	-	75	188	-	62	81	-	-
3	Suff	VLA00-0 45753		-	49	67	-	90	97	-	48	65	-	-
4	Oxon	VLA00-0 45776		+++	839	166	(+)	132	75	-	99	77	+	-
5	W.Midland	VLA00-0 45599		-	52	65	-	63	101	-	63	66	-	-
6	Berks	VLA00-0 45606		-	54	65	-	48	26	-	70	55	-	-
7	Denmark	VLA00-0 45760		-	71	83	-	73	102	-	70	60	-	-
8	W.Susses	VLA00-0 45758		?	113	107	-	56	65	-	58	36	-	-
9	W.Susses	VLA00-0 45757		?	105	102	-	41	85	(+)	126	47	-	-

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
10	Suff	VLA00-0 45751		(+)	124	114	-	80	69	(+)	128	88	-	-
12	Berks	VLA00-0 45775		?	131	125	-	60	133	-	65	47	-	-
13	Suff	VLA00-0 45754		-	142	78	-	55	82	-	75	80	-	-
14	Suff	VLA00-0 45822		-	118	29	-	73	54	-	51	42	-	-
15	Suff	VLA00-0 45909		-	45	41	-	48	55	-	43	8	-	-
16	Berks	VLA00-0 45771		-	28	27	-	76	64	-	70	58	-	-
17	Suffolk	VLA00-0 45818		-	33	40	-	66	60	-	61	39	-	-
18	Suff	VLA00-0 45893		-	43	35	-	28	89	-	61	26	-	-
19	Berks	VLA00-0 46141		-	32	36	-	28	27	-	47	30	-	-
20	Berks	VLA00-0 5603		-	35	47	-	20	44	-	82	36	-	-
21	Suff	VLA00-0 45910		-	29	47	-	18	37	?	101	85	-	-
22	Suff	VLA00-0 46130		-	35	20	-	65	79	-	53	36	-	-
23	Berks	VLA00-0 45854		-	26	32	-	73	61	++	333	111	-	-

Appendix III-b:

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
24	Berks	VLA00-0 45852		-	20	38	-	98	66	?	110	61	-	-
25	Berks	VLA00-0 45855		-	32	38	?	106	172	+	170	106	-	-
26	Leics	VLA00-0 45857		-	32	54	-	77	82	-	91	32	-	-
27	Kent	VLA00-0 45979		-	26	3	-	44	53	-	37	31	-	-
28	Suff	VLA00-0 45889	1997	-	26	26	-	2	113	+	169	123	+	-
29	Suff	VLA00-0 45907		-	28	12	-	35	41	-	72	43	-	-
30	Suff	VLA00-0 46129		-	38	22	-	73	122	-	71	32	-	-
31	N.Yorks	VLA00-0 46140		-	25	29	-	74	61	-	75	30	-	-
32	Suff	VLA00-0 45904	1998	-	20	16	-	51	60	(+)	129	71	-	-
33	Suff	VLA00-0 45890		-	16	16	-	61	60	-	58	53	-	-
34	Kent	VLA00-0 45865		-	67	48	+	239	219	++	302	220	-	-
35	Suff	VLA00-0 45891		-	25	27	-	46	51	-	97	33	-	-
36	Suff	VLA00-0 45988		-	27	22	-	31	48	-	29	26	-	-

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
37	Suff	VLA00-0 45911		+++	913	689	++	567	488	++	463	348	+	-
38	Shrops	VLA00-0 45864		-	37	21	-	32	73	-	69	71	-	-
39	Suff	VLA00-0 45901		+++	1425	1179	+++	980	###	+++	809	567	+	+
40	Suff	VLA00-0 45902		-	47	21	-	48	63	-	46	68	-	-
46	Berks	VLA00-0 45853		-	64	35	(+)	131	169	?	105	96	-	-
47	Berks	VLA00-0 45871		+++	600	264	++	309	620	++	800	400	+	-
48	Hants	VLA00-0 45867		-	25	45	-	29	18	-	75	39	-	-
49	Suff	VLA00-0 45824		-	15	10	-	59	70	-	82	86	-	-
50	Kent	VLA00-0 45866		-	18	16	-	54	108	-	92	92	-	-
51	Suff	VLA00-0 45816		+	230	100	++	308	156	++	323	215	-	+
52	Hampshire	VLA00-0 45761		-	15	10	-	50	59	-	74	42	-	-
53	Suff	VLA00-0 45817		-	36	19	-	80	92	-	72	56	-	-
54	Suff	VLA00-0 45888	1998	-	20	38	-	37	20	-	56	57	-	-

Appendix III-b:

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
55	Suff	VLA00-0 46204		-	12	1	-	22	40	-	65	54	-	-
56	Suff	VLA00-0 46201		+++	14	15	-	44	19	-	56	50	-	-
57	Rep. Ireland	VLA00-0 45609		-	832	467	++	376	487	++	431	515	+	-
58	Berks	VLA00-0 45607		-	57	54	-	70	82	-	49	45	-	-
59	Berks	VLA00-0 45764		-	96	80	-	35	36	-	81	17	-	-
60	Suff	VLA00-0 46208	1998	-	77	15	-	12	20	-	42	65	-	-
61	Suff	VLA00-0 46207		-	68	13	-	42	52	-	50	23	-	-
62	Berks	VLA00-0 45604		-	75	1	-	34	63	-	72	84	-	-
63	Berks	VLA00-0 45608		(+)	121	15	-	47	77	-	72	22	-	-
64	Suff	VLA00-0 45820		-	73	49	-	33	17	-	90	60	-	-
65	w.Midlands	VLA00-0 45600		-	58	48	-	36	4	-	36	34	-	-
66	Oxon	VLA00-0 45601		-	86	17		32	29	-	96	122	-	-
67	Suff.	VLA00-0 45827		-	68	2	-	26	14	-	87	60	-	-

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
68	Suff.	VLA00-0 45905		-	80	70	-	28	50	-	96	33	-	-
69	Suff	VLA00-0 46116		(+)	127	57	-	56	112	?	103	87	-	+
70	Oxfordshire	VLA00-0 45772		-	74	91	-	76	197	-	28	47	-	-
71	Oxfordshire	VLA00-0 45774		-	53	44	-	56	118	-	39	30	-	-
72	Berks	VLA00-0 45763		-	59	61	-	89	261	-	97	62	-	-
73	Berks	VLA00-0 45602		-	9	75	-	47	120	-	88	62	-	-
74	Suff	VLA00-0 45819		-	17	63	?	116	140	-	66	91	-	-
75	Suff	VLA00-0 45821		-	51	58	-	96	275	-	98	34	-	-
76	Suff	VLA00-0 46218		-	88	47	-	71	52	-	49	41	-	-
77	Suff	VLA00-0 46216		-	47	48	-	61	25	-	35	41	-	-
78	Suff	VLA00-0 46206	1998	-	61	54	-	56	93	-	55	42	-	-
79	Suff	VLA00-0 46205		-	61	53	-	48	46	-	52	54	-	-
80	Rep. Ireland	VLA00-0 46194		+++	803	548	++	520	451	++	313	217	+	-

Appendix III-b:

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
81	Suff	VLA00-0 46217		-	50	28	-	57	65	-	4	7	-	-
82	Suff	VLA00-0 46131		-	30	17	-	65	144	-	80	115	-	-
83	Suff	VLA00-0 45892		-	27	52	-	53	50	-	64	23	-	-
84	Rep. Ireland	VLA00-0 46193		-	55	7	-	74	43	-	60	48	-	-
85	Suff	VLA00-0 46219		-	44	29	?	106	199	-	65	84	-	-
86	Suff	VLA00-0 45906		-	39	24	-	91	92	-	10	21	-	-
87	Suff	VLA00-0 45884	1999	-	40	27	-	86	66	-	59	49	-	-
88	Berks	VLA00-0 45879		-	34	22	-	40	43	-	38	39	-	-
89	Suff	VLA00-0 45826		-	30	24	-	56	81	-	83	95	-	-
90	Suff	VLA00-0 45900		-	42	21	-	94	126	?	106	52	-	-
91	Suff	VLA00-0 45899		-	38	45	-	77	143	-	80	7	-	-
92	Suff	VLA00-0 45887	1998	-	24	20	-	71	71	?	106	52	-	-
93	Leics	VLA00-0 46118		++	465	453	++	402	504	++	455	167	-	+

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
94	Suff	VLA00-0 45898		-	21	23	-	84	130	-	79	27	-	-
95	Suff	VLA00-0 45894	1998	-	24	18	-	76	129	-	95	53	-	-
96	Wilts	VLA00-0 45750		-	24	26	-	59	139	-	87	63	-	-
97	Rep. Ireland	VLA00-0 59876		+++	907	1190	++++	###	###	++++	###	1214	+	+
98	N.Yorks	VLA00-0 59640		-	28	20	-	67	97	-	78	41	-	-
99	Rep. Ireland	VLA00-0 59483		-	27	23	-	82	143	(+)	143	83	-	-
100	Suff	VLA00-0 59498	2000	-	22	12	?	101	103	-	75	28	-	-
101	Rep. Ireland	VLA00-0 59857		+++	690	565	++	373	344	+	282	22	+	-
102	Rep. Ireland	VLA00-0 59886		-	30	14	-	48	44	-	25	2	-	-
103	Suff	VLA00-0 60045	2000	-	91	98	-	50	90	-	45	7	-	-
104	Rep. Ireland	VLA00-0 59885		-	67	64	-	48	73	-	41	17	-	-
105	Rep. Ireland	VLA00-0 59858		-	44	85	-	89	68	-	28	65	-	-
106	Rep. Ireland	VLA00-0 59482		-	97	52	-	49	110	-	78	6	-	-

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
107	Yorks	VLA00-0 59494		-	69	71	-	43	68	-	59	16	-	-
108	Rep. Ireland	VLA00-0 59484		-	58	70	-	57	164	-	64	48	-	-
109	Rep. Ireland	VLA00-0 59497		-	76	59	-	64	149	-	78	7	-	-
110	N.Yorks	VLA00-0 59642		-	69	57	-	67	82	-	37	51	-	-
111	Dorset	VLA00-0 59489		-	63	64	-	89	115	-	73	52	-	-
112	Suffolk	VLA00-0 59805		-	99	79	-	52	69	-	33	16	-	-
113	Rep. Ireland	VLA00-0 59859		++++	1188	901	++	596	885	+++	605	900	+	+
114	Rep. Ireland	VLA00-0 59877		-	81	111	-	83	129	(+)	135	85	-	-
115	Rep. Ireland	VLA00-0 59879		-	44	63	?	98	470	-	99	100	-	-
116	Suff	VLA00-0 60049	2000	-	33	35	-	114	208	-	40	20	-	-
117	Rep. Ireland	VLA00-0 59855											-	-
118	Glos	VLA00-0 59799		++++	1508	1534	+++	952	###	+++	821	650	-	+
119	Suff	VLA00-0 60047	2000	-	85	54	-	53	193	-	33	37	-	-

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
120	Suff	VLA00-0 59653		-	59	43	-	69	133	-	47	35	-	-
121	Glos	VLA00-0 59798		-	35	80	-	77	110	-	51	17	-	-
122	Suff	VLA00-0 59804		-	83	47	+	208	80	+	172	63	-	-
123	Suff	VLA00-0 59652	1999	-	80	47	(+)	126	260	-	43	96	-	-
124	Rep. Ireland	VLA00-0 59889		++++	1329	1122	++	524	921	+++	727	501	+	+
125	Rep. Ireland	VLA00-0 59862		-	34	52	-	72	62	-	33	21	-	-
126	Rep. Ireland	VLA00-0 59861		-	74	61	-	96	105	-	35	142	-	-
127	Rep. Ireland	VLA00-0 69868		-	69	56	-	85	120	-			-	-
128	Rep. Ireland	VLA00-0 59871		+	165	76	(+)	139	201	?	113	77	-	+
129	Rep. Ireland	VLA00-0 59854		++	489	389	++	313	654	++	382	342	-	-
130	Rep. Ireland	VLA00-0 60124		+++	926	730	++	423	831	++	548	656	-	-
131	Rep. Ireland	VLA00-0 60123		++++	2532	2405	++++	###	###	++++	###	982	+	-
132	Denbigh	VLA00-0 59649		-	70	42	-	50	77	-	22	27	-	-

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
133	Rep. Ireland	VLA00-0 59867		-	81	43	-	45	120	-	64	46	-	-
134	Rep. Ireland	VLA00-0 59481		-	60	46	-	49	157	-	27	30	-	-
135	E.Lothian	VLA00-0 59802		-	72	39	-	45	109	(+)	121	78	-	-
136	Rep. Ireland	VLA00-0 59866		-	86	61	-	37	161	(+)	141	108	-	-
137	Rep. Ireland	VLA00-0 59860		-	52	46	-	36	48	-	52	38	-	-
138	Rep. Ireland	VLA00-0 59888		+++	765	386	++	368	636	++	450	641	-	-
139	Suff	VLA00-0 59803		-	44	53	-	48	60	-	63	51	-	-
140	Suff	VLA00-0 59806		-	44	48	-	4	60	-	56	42	-	-
141	Rep. Ireland	VLA00-0 59880		?	110	60	++	323	550	++	194	194	-	-
142	Powys	VLA00-0 59869		++++	2312	2175	+++	604	859	+++	791	909	+	+
143	Suff	VLA00-0 60049	2000	-	57	66	-	43	66	-	50	26	-	-
144	Rep. Ireland	VLA00-0 59881		-	54	36	-	42	27	-	60	46	-	-
145	Rep. Ireland	VLA00-0 59891		++++	1856	1542	+++	723	765	+++	804	433	+	-

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
146	Rep. Ireland	VLA00-0 59884		-	63	48	-	25	41	-	61	33	-	-
147	Rep. Ireland	VLA00-0 59882		-	64	50	-	48	68	-	75	36	-	-
148	Suff	VLA00-0 60044	2000	-	57	56	-	24	27	-	27	33	-	-
149	Suff	VLA00-0 60050	2000	-	54	49	-	19	40	-	34	38	-	-
150	Rep. Ireland	VLA00-0 59875		++++	1278	652	+	274	311	++	321	97	-	+
151	Glos	VLA00-0 59795		-	87	58	-	30	56	-	39	48	-	-
152	Essex	VLA00-0 59500		-	94	76	-	61	83	-	9	8	-	-
153	Yorks	VLA00-0 59499		-	81	57	+	239	152	-	62	77	-	-
154	Sussex	VLA00-0 59495		++	559	125	++	519	359	++	572	160	-	-
155	Cornwall	VLA00-0 59545		-	84	51	-	42	58	-	46	52	-	-
156	Rep. Ireland	VLA00-0 59544		-	70	39	-	88	94	-	51	29	-	-
157	Rep. Ireland	VLA00-0 59873		++++	2597	2060	++++	###	###	+++	901	327	+	-
158	Rep. Ireland	VLA00-0 59874		?	101	87	-	50	76	-	79	39	-	-

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
159	Rep. Ireland	VLA00-0 59863		+++	853	615	++	450	592	+	290	73	+	+
160	Rep. Ireland	VLA00-0 59864		+	179	104	-	60	111	-	70	54	-	-
161	Suff	VLA00-0 60052	2000	+	283	116	++	462	478	+	290	103	-	-
162	Israel	VLA00-0 59945		-	32	41	-	85	98	-	78	77	-	-
163	Rep. Ireland	VLA00-0 59872		-	33	38	-	79	133	-	84	71	-	-
164	Suff	VLA00-0 60051	2000	-	41	37	-	55	141	-	67	90	-	-
165	Rep. Ireland	VLA00-0 59883		-	41	49	-	82	113	-	81	60	-	-
166	Suff	VLA00-0 60042	2000	-	38	33	-	87	108	-	97	58	-	-
167	Suff	VLA00-0 60142		-	42	39	-	99	115	-	97	5	-	-
168	Glos	VLA00-0 60147		++++	2210	1184	++++	###	###	++	326	445	+	+
169	Suff	VLA00-0 60048	2000	-	72	65	-	97	117	-	53	39	-	-
170	Suff	VLA00-0 60043	2000	+	55	44	-	66	72	-	43	34	-	-
171	Rep. Ireland	VLA00-0 59887		-	79	42	-	59	104	-	31	49	-	-

Horse samples

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
172	Glos	VLA00-0 60150		++++	2732	2033	++++	###	###	+++	681	891	+	-
173	Suff	VLA00-0 60053	2000	-	66	41	-	10	20	-	91	35	-	-
174	Glos	VLA00-0 60149		++++	1198	383	++	524	338	++	410	409	-	-
175	Glos	VLA00-0 60148		++++	1119	431	+++	700	397	++	449	351	+	+
176	Cornwall	VLA00-0 59547		-	74	37	-	66	148	?	104	83	-	-
177	Lancs	VLA00-0 59928		-	72	35	?	115	95	-	54	75	-	-
178	Suff	VLA00-0 59801		-	66	48	-	55	95	-	58	76	-	-
179	Cheshire	VLA00-0 59549		-	65	38	-	59	60	-	43	49	-	-
180	Rep. Ireland	VLA00-0 59890		-	54	51	-	34	45	-	41	41	-	-
181	Suff	VLA00-0 60055	2000	-	46	41	-	60	67	-	57	42	-	-
182	Rep. Ireland	VLA00-0 59870		-	41	34	-	73	110	-	86	91	-	-
183	Rep. Ireland	VLA00-0 59878		-	32	32	-	62	44	-	50	58	-	-
184	Suff	VLA00-0 59800		-	34	29	-	60	44	-	47	52	-	-

N.	Vets County	Ref.	DOB	Ag	1	2	CIC	1	2	Ab	1	2	IFA	PCR
185	Lincs	VLA00-0 59550		-	35	28	-	41	55	-	54	47	-	-
186	Berks	VLA00-0 59551		-	40	28	-	42	48	-	37	50	-	-
187	Rep. Ireland	VLA00-0 59865		-	37	29	-	45	43	-	25	36	-	-
188	Cornwall	VLA00-0 59546		-	33	29	-	69	82	-	69	58	-	-
189	Suff	VLA00-0 60054	2000	-	45	32	-	44	59	-	87	60	-	-
190	Rep. Ireland	VLA00-0 60122		++++	2308	33	++++	###	###	+++	623	543	+	-
191	Cornwall	VLA00-0 59548		-	78	54	-	60	97	-	58	103	-	-
192	Berks	VLA00-0 59501		-	49	40	-	55	83	-	28	40	-	
193	Denbigh	VLA00-0 596650		-	92	46	(+)	121	217	?	106	73	-	-
194	Norfolk	VLA00-0 59496		-	75	49	-	59	54	-	56	67	-	-
195	Glos	VLA00-0 60151		+	167	61	(+)	149	290	-	75	84	-	-
196	Rep. Ireland	VLA00-0 59856		-	85	51	-	85	71	-	56	110	-	-

Table III-b.1: Ag = antigen test results; Ab = antibody test results. 1. = OD1; 2. = OD2. Note that OD values are presented without the decimal places. OD value less than 0.079 were negative; OD values between 0.079 and 0.149 were all retested.

BDV sequences

Number	Origin	Sequence	% homology	Gaps
1	Bode's protocol rabbit spleen cells (YRS)	ACACAAAGGAGCCTACCCAGGGTTGGCCGTTAATCCAATCTATAGCCTCATGTGGATTAA ACATCTGGAGTAGTGTAGCAGTCTCACCATGGGATGGCCGGTTTAAGGCTGCCATCATAG TTTTAAACCTTTTCTTGATCTGCTCGGCTCCTGCTTTGATCTTAGACGACGATCCTATCAC AACCCCTATTAGTAATGAGCAACAATGGCTGAAGATAGAGGAGATCTCCAGCTCGGTGAG ATCACGCTGCGTCGTCTTTTCCCCGTAAAACTTCGCAGTCTTAACAACAGTCTGTTCTCCAC GCGTGACAGGCGTCGACAGGTAGGATTCACGAGGCACCCCTCCGTGAACAAACGCAGCG TGCAGGTCCTGGGATTAGCAAACATAGAAACACAAGGCT AGGCGTTA CTGTGTGAA	99 (417/418)	1/418
2	rabbit spleen cells RT-PCR by Schwartz	GAGACAACACAAAGGAGCCTACCCAGGGTTGGCCGTTAATCCAATCTATAGCCTCATGTGG ATTAAACATCTGGAGTAGTGTAGCAGTCTCACCATGGGATGGCCGGTTTAAGGCTGCCATCA TAGTTTTAAACCTTTTCTTGATCTGCTCGGCTCCTGCTTTGATCTTAGACGACGATCCTATCAC AACCCCTATTAGTAATGAGCAACAATGGCTGAAGATAGAGGAGATCTCCAGCTCGGTGAGAT CACGCTGCGTCGTCTTTTCCCCGTAAAACTTCGCAGTCTTAACAACAGTCTGTTCTCCACGCGT GACAGGCGTCGACAGGTAGGATTCACGAGGCACCCCTCCGTGAACAAACGCAGCGTGCAGTCC TGGGATTAGCAAACATAGAAACACAA	100	0/402
3	rabbit spleen cells (186 and 606)	GTGGCCGTTAATCCAATCTATAGCCTCATGTGGATTAAACATCTGGAGTAGTGTAGCAGTCTC ACCATGGGATGGCCGGTTTAAGGCTGCCATCATAGTTTTAAACCTTTTCTTGATCTGCTCGGCT CCTGCTTTGATCTTAGACGACGATCCTATCACAACCCCTATTAGTAATGAGCAACAATGGCTGA AGATAGAGGAGATCTCCAGCTCGGTGAGATCACGCTGCGTCGTCTTTTCCCCGTAAAACTTCG CAGTCTTAACAACAGTCTGTTCTCCACGCGTGACAGGCGTCGACAGGTAGGATTCACGAGGCA CCCCTCCGTGAACAAACGCAGCGTGCAGTCCTGGGATTAGCAAACATA	100	0/364

Number	Origin	Sequence	% homology	Gaps
4	patient 43 (group1)	ACACAAAGGAGCCTACCCAGGGTTGGCCGTTAATCCAATCTATAGCCTCATGTGGATTAAACATC TGGAGTAGTGTAGCAGTCTCACCATGGGATGGCCGGTTTAAGGCTGCCATCATAGTTTTAAACCT TTTCTTGATCTGCTCGGCTCCTGCTTTGATCTTAGACGACGATCCTATCACAACCCCTATTAGTAAT GAGCAACAATGGCTGAAGATAGAGGAGATCTCCAGCTCGGTGAGATCACGCTGCGTCGTCTTTTC CCCGTAAAACTTCGCAGTCTTAACAACAGTCTGTTCTCCACGCGTGACAGGCGTCGACAGGTAGGAT TCACGAGGCACCCCTCCGTGAACAAACGCAGCCGTGCAGTCCTGGGATTAGCAAACATA	99 387/388	0/388
5	patient 58 (Group1)	ACAACACAAAGGAGCCTACCCAGGGTTGGCCGTTAATCCAATCTATAGCCTCATGTGGATTAAACA TCTGGAGTAGTGTAGCAGTCTCACCATGGGATGGCCGGTTTAAGGCTGCCATCATAGTTTTAAACCT TTTCTTGATCTGCTCGGCTCCTGCTTTGATCTTAGACGACGATCCTATCACAACCCCTATTAGTAATGAG CAACAATGGCTGAAGATAGAGGAGATCTCCAGCTCGGTGAGATCACGCTGCGTCGTCTTTTCCCCGTA AAACTTCGCAGTCTTAACAACAGTCTGTTCTCCACGCGTGACAGGCGTCGACAGGTAGGATTCACGAG GCACCCCTCCGTGAACAAACGCAGCGTGCAGTCCTGGGATTAGCAAACATA	100 390	0/390
6	patient 2 (Group1)	CAACACAAAGGAGCCTACCCAGGGTTGGCCGTTAATCCAATCTATAGCCTCATGTGGATTAAACATCT GGAGTAGTGTAGCAGTCTCACCATGGGATGGCCGGTTTAAGGCTGCCATCATAGTTTTAAACCTTTTCT TGATCTGCTCGGCTCCTGCTTTGATCTTAGACGACGATCCTATCACAACCCCTATTAGTAATGAGCAAC AATGGCTGAAGATAGAGGAGATCTCCAGCTCGGTGAGATCACGCTGCGTCGTCTTTTCCCCGTAAAAC TTCGCAGTCTTAACAACAGTCTGTTCTCCACGCGTGACAGGCGTCGACAGGTAGGATTCACGAGGCAC CCCTCCGTGAACAAACGCAGCGTGCAAGTCCTGGGATTAGCAAACATAG	99 390/391	1 390/391

Table V.1: Comparison of p40 nucleotide sequences between BDVP1 isolate derived BDV sequences. Total RNA extracted from OL cells persistently infected with the BDV Pte isolate was amplified by RT-PCR using the p40 primers described previously.

Variation of BDV detection by RT-PCR-I

Disease	Tissue	Disease	Prevalence (‰)	Controls	(‰)	References
Psychiatric	PBMC	(4/6)	66.7	(0/10)	0	Bode *et al.*, 1995
(various)	PBMC	(5/12)	41.7	(0/23)	0	Sauder *et al.*,1996
	PBMC	(22/60)	37	(8/172)	4.7	Kishi *et al.*, 1995
	PBMC-co-culture	(3/32)	9.4	(0/5)	0	Bode *et al.*, 1996; de la Torre *et al.*, 1996
Affective	PBMC	(1/3)	33.3	(0/23)	0	Sauder *et al.*,1996
	PBMC	(1/6)	16.7	(0/36)	0	Igata-Yi *et al.*, 1996
	PBMC	(0/9)	0			Sauder *et al.*,1996
Schizophrenia	PBMC	(7/11)	63.6	(0/23)	0	Sauder *et al.*,1996
	CSF	(0/48)	0	(0/9)	0	Sierra-Honigman *et al.*, 1995
	PBMC	(0/9)	0	(0/9)	0	Sierra-Honigman *et al.*, 1995
	PBMC	(0/26)	0			Rich *et al.*, In press
CFS	PBMC	(3/25)	12			Nakaya *et al.*, 1995
Hippocampal sclerosis	Brain	(4/5)	80			De la Torre *et al.*, 1996

Table V.1: Borna disease virus nucleic acid in patients with various diseases. Extracted from Hatalski et al., 1997 with small modifications. Abbreviations: PBMC, peripheral blood mononuclear cells; CSF, cerebrospinal fluid; CFS, chronic fatigue syndrome.

Variation of BDV detection by RT-PCR-II

Disease	Sample	Comments	Reference
Psychiatric	PBMC	67% vs 0%a (0%-3.6% divergence at 'P' gene)	
Psychiatric	PBMC	37%	Kishi *et al.*, 1995
Blood donor	PBMC	5%	Kishi *et al.*, 1995
Schizophrenia	Brain/CSF/PBMC	0% vs 0%	Sierra-Honigmann *et al.*, 1995
Schizophrenia	PBMC	3 positive cases (4.2%-9.3% divergence at 'P' gene)	Kishi *et al.*, 1996
Psychiatric	PBMC	42% vs 0% (0%-4% divergence at 'P' gene)	Sauder *et al.*, 1996
Affective disorders	PBMC	33% vs 0%	
Schizophrenia	PBMC	64% vs 0%	
Psychiatric	PBMC	9% vs 0% (0.07%-0.83% divergence at 'P' gene)	Bode *et al.*, 1996 de la Torre *et al.*, 1996
Affective disorders	PBMC	17% vs 0%	Igata-Yi *et al.*, 1996
Schizophrenia	PBMC	10% vs 0%	
CFS	PBMC	12% (6.0%-14% divergence at 'P' gene)	Nakaya *et al.*, 1996
CFS	PBMC	12.3% vs 4.7%	Kitani *et al.*, 1996

Disease	Sample	Comments	Reference
Hippocampal sclerosis	Brain	80% Confirmed the data of de la Torre *et al.*, 1996	de la Torre *et al.*, 1996 Czygan *et al.*, 1999
Psychiatric	PBMC	2% vs 0%	Kubo *et al.*, 1997
Psychiatric/Schizophrenia	PBMC	0% vs 0%	Richt *et al.*, 1997
Psychiatric/Schizophrenia	Whole blood/PBMC	0%	Lieb *et al.*, 1997
Schizophrenia	Brain	53%	Salvatore *et al.*, 1997
Affective disorders	Brain	40%	
Alzheimer's disease	Brain	0%	
Parkinson's disease	Brain	0%	
Multiple sclerosis	Brain	0%	
No neurological control	Brain	0%	
Schizophrenia	Brain	33%	Haga *et al.*, 1997
Parkinson's disease	Brain	16.7%	
Healthy control	Brain	6.5%	
Healthy control	Brain	6.7%	Haga *et al.*, 1997
Psychiatric	PBMC	one positive case	Planz *et al.*, 1998
Schizophrenia	PBMC	4% vs 2% (0%-5.1% divergence at 'P' gene)	Iwata *et al.*, 1998

Disease	Sample	Comments	Reference
Schizophrenia	PBMC	13.5% vs 1.4%	Chen *et al.*, 1999
CFS	PBMC	86% in 2 family clusters	Nakaya *et al.*, 1999
Psychiatric (3 patients)	Whole blood Granulocytes PBMC	100% vs 0% 100% vs 0% 67% vs 0%	Plantz *et al.*, 1999
Affective disorders	PBMC	0%	Kim *et al.*, 1999
CFS	PBMC	0%	Evengard *et al.*, 1999
HIV-positive	PBMC/CSF	0% in patients with various neurological diseases 0% in patients with AIDS dementia complexes	Backmann *et al.*, 1999
Affective disorders Schizophrenia	Brain Brain	0% 0%	Czygan *et al.*, 1999
Psychiatric	PBMC	37% vs 15.4%	Vahlenkamp *et al.*, 2000
Schizophrenia	PBMC	1.8% vs 0.6%	Tsuji *et al.*, 2000
CFS	PBMC	One positive case (3.8% divergence at 'P' gene)	Nowotny *et al.*, 2000
Fibromyalgia	Serum/CSF	0%	Wittrup *et al.*, 2000
Schizophrenia	Brain	25% vs 0%	Nakamura *et al.*, 2000

Table V.2: Borna disease virus nucleic acid in patients with various diseases. Extracted from Ykuta *et al.*, 2002: CFS=chronic fatigue syndrome; PBMC=peripheral blood mononuclear cell; CSF=cerebrospinal fluid.

Variation of BDV anti-antibody detection by serology

Disease	Assay	Comments	Reference
Psychiatric	IFA	6% vs 0%b	Rott *et al.*, 1985
Affective disorders	IFA	4% vs 0%	
Affective disorders	IFA	4.5% vs 0%	Amsterdam *et al.*, 1985
Psychiatric	IFA	2% vs 2%	Bode *et al.*, 1988
HIV-positive	IFA	7.8% vs 2.0%	
Psychiatric/neurological diseases	IFA/WB	4%-7% vs 1%	Rott *et al.*, 1991
Chronic diseases	IFA/IP	13%-14% vs 2%	Bode *et al.*, 1992
CFS	IFA	0%	Bode *et al.*, 1992
Psychiatric	IFA	Significantly higher than controls (surgical patients)	Bechter *et al.*, 1992
Psychiatric/Affective disorders	IFA	20% (increased from 2%-4% by follow-up studies)	Bode *et al.*, 1993
Affective disorders	WB	38% vs 16% in 'N'; 12% vs 4% in 'P'	Fu *et al.*, 1993
Psychiatric	IFA	50%	Bode *et al.*, 1995
Psychiatric	WB	30%	Kishi *et al.*, 1995

Disease	Assay	Comments	Reference
Schizophrenia	WB	8.9% vs 0% in 'N'; 27.8% vs 20% in 'P'	Waltrip *et al.*, 1995
Schizophrenia	IFA	16.7% vs 15%	
Psychiatric		Increased CSF/serum index for anti-BDV antibodies in 10.5-29.0% (according to different methodological limits) of BDV-seropositive patients	Bechter *et al.*, 1995
Psychiatric	WB	14% vs 1.5%	Sauder *et al.*, 1996
Affective disorders	WB	12% vs 1.5%	
Psychiatric	IFA	24% vs 11%	Igata-Yi *et al.*, 1996
CFS	WB	24%	Nakaya *et al.*, 1996
HIV-positive	ELISA	38%-48% in STD patients infected with subtype E 8.3% in prostitutes infected with subtype E 0% in pregnant women infected with subtype E 0% in intravenous drug users infected with subtype B 0% in HIV-1-negative individuals	Auwanit *et al.*, 1996
Multiple sclerosis	IFA	0%	Kitze *et al.*, 1996
Deficit schizophrenia	WB	33.3%	Waltrip *et al.*, 1997
Nondeficit schizophrenia	WB	8.2%	
Schizophrenia	WB	20%	Richt *et al.*, 1997
Schizophrenia	WB	45%	Iwahashi *et al.*, 1997

Disease	Assay	Comments	Reference
Psychiatric	IFA/WB	0%	Kubo *et al.*, 1997
Affective disorders	IFA/WB	0%-0.8%	
Schizophrenia	IFA/WB	0%-1%	
Controls	IFA/WB	0%	
Schizophrenia	RT-ELISA	0% vs 0%	Horimoto *et al.*, 1997
Major depression		6.3% in CSF	Deuschule *et al.*, 1998
Multiple sclerosis		0% in CSF	
Schizophrenia	ECLIA	3.08%	Yamaguchi *et al.*, 1999
Mood disorders	ECLIA	3.59%	
Other psychiatric	ECLIA	0%	
Neurological disorders	ECLIA	0%	
CFS	ECLIA	0%	
HIV-positive	ECLIA	1.18%	
Autoimmune	ECLIA	0%	
Blood donors	ECLIA	1.09%	
Multitransfusion	ECLIA	4.55%	
HIV-positive	IFA	12.5% in patients with various neurological disorders; 8.0% in patients with AIDS dementia complex	Backmann *et al.*, 1999

Disease	Assay	Comments	Reference
Schizophrenia	WB	12.1%	Chen *et al.*, 1999
Family members	WB	12.1%	
Mental health workers	WB	9.8%	
Controls	WB	2.9%	
CFS	WB	100% in 2 family clusters	Nakaya *et al.*, 1999
CFS	ELISA/WB	0% vs 0%	Evengard *et al.*, 1999
Psychiatric	IFA	17.4% vs 0%	Valenkamp *et al.*, 2000
Psychiatric/Schizophrenia	WB	0%	Tsuji *et al.*, 2000
Psychiatric	ECLIA	10.2% (recent onset of disease)	Rybakowski *et al.*, 2001
	ECLIA	1.6% (>1 year illness)	
Mood disorders	TCPR	9%	Fukuda *et al.*, 2001
Schizophrenia	TCPR	4%	
Blood donors	TCPR	2%	

Table V.3: Borna disease anti-antibodies in patients with various diseases. Extracted from Ykuta et al., 2002. HIV=human immunodeficiency virus; CFS=chronic fatigue syndrome; IFA=immunofluorescence assay; WB=Western blotting; IP=immunoprecipitation; ELISA=enzyme-linked immunosorbent assay; RT-ELISA=reverse-type ELISA; ECLIA=electrochemiluminescence immunoassay; TCPR=T-cell proliferative response; CSF=cerebrospinal fluid; STD=sexually transmitted disease. a Chronic progressive diseases of the brain and the immune system. b Controls.

Detection of BDV antigens in subjects with various diseases

Disease	Tissue	Assay	Comments	Reference
Psychiatric	PBMC	Flow cytometry	67%	Bode *et al.*, 1995
Hippocampal sclerosis	Brain	IHC	80%	de la Torre *et al.*, 1996
Major depression	CSF	ELISA	9.4%	Deuschule *et al.*, 1998
Multiple sclerosis	CSF	ELISA	10.5%	
Depression	PBMC	Flow cytometry	37.2% vs 1.01%a	Ferszt *et al.*, 1999
Schizophrenia	Brain	IHC	25% vs 0%	Nakamura *et al.*, 2000
Affective disorders	Plasma	ELISA	62% in Berlin and 52% in Hanover for CICs	Bode *et al.*, 2001
Healthy controls	Plasma	ELISA	24% for CICs	

Table V.4: Borna disease antigens in patients with various diseases. Extracted from Ykuta et al., 2002.PBMC=peripheral blood mononuclear cell; CSF=cerebrospinal fluid; IHC=immunohistochemistry; ELISA=enzyme-linked immunosorbent assay; CIC=BDV-specific circulating immune complex. a Controls.

AGENDA ITEM

JOINT ETHICS COMMITTEE

Newcastle and North Tyneside Health Authority
University of Newcastle upon Tyne
University of Northumbria at Newcastle

Form of Application for Ethical Approval for Research Project
Notes

(i) This form must be typed or word-processed in accordance with the Information/Guidance for Applicants document and, complete with associated paperwork, must be received by the Administrator to the Joint Ethics Committee at least 17 days before the meeting held on 2nd Tuesday of the month. A contrasting font, emboldening or BLOCK CAPITALS must be used to clearly distinguish applicants' answers from the original questions on the form.

(ii) A 150 word summary of the research protocol in lay terms must be included on page 4 of this form.

(iii) Applicants must answer all questions and ensure that the form is signed by the Applicant and normally also by the responsible Clinician on page 4, otherwise consideration of the application by the Ethics Committee will be delayed.

(iv) Where applicable, the agreement to the proposed study, of all medically qualified consultants and senior non-medical health professionals with responsibility for patients, whose patients may be involved, must be recorded.

1. Title of project:

Borna Disease Virus - A UK perspective (This is the title of my PhD Project), but can we still use the " the prevalence of BDV in patients with mood disorders and health controls for now?
2.a) Applicant/Lead Researcher:
Stuart Watson

Appointment held and Institution where based:
Specialist Registrar
Royal Victoria Infirmary

b) Research Team:
Supervisor of Research Project
Jennifer Higham

Person responsible for the relevant service to patients e.g. Consultant / GP / Health Care Professional:

Professor Allan Young, The Department of Psychiatry, Leazes Wing, RVI

Names and Appointments of ALL other members of the research group

Stuart Watson - Royal Victoria Infirmary
Maria Chimpolo - University of Northumbria
Jennifer Higham - University of Northumbria

3. In what location will the subjects be studied:
School of Applied and Molecular Biology
University of Northumbria, Newcastle
Ellison Building

4. Will the study involve subjects who are not under the care of clinicians in the Newcastle & North Tyneside health district?

Yes

5. Categories of subjects including number, age, sex and whether in-patient or out-patient:
a) Patients:

bipolar patients

b) Patient controls:
0

c) Healthy volunteers

6. In the case of healthy volunteers how and from where will they be sought?:
By local advertisement

7. Communication with General Practitioner:
a) how will the GP be notified of participation in study?

By letter (enclosed)
b) if not before commencement, what is the reason for this?

8. What steps will be taken to ensure that individual subjects/patients are not entered repeatedly into research projects/trials?
We will ask them.

9. What significant discomfort (physical or psychological), inconvenience or danger will be caused?

Inconvenience of time and pain of venepuncture

10. What particular ethical problems do you think there are in the proposed study? - What measures will be adopted to protect patient confidentiality?

We are examining the influence of Borna Disease Virus on the pathophysiology of bipolar disorder (manic-depression). Subjects will be able to be made aware of their Borna Disease status. Borna is however, common in the general population and its presence or absence does not require treatment or concern. We will offer full consultation with subjects about their Borna Disease status if appropriate.

11. How will the design of the study ensure scientific validity:
describe and explain the methodology to be used

The results obtained from the previous study with patients under the care of clinicians in the Newcastle and North Tyneside health district, supported the evidence about the presence of the virus in the UK (unpublished data).
The methodology to be used is one already in use in UK, Germany, Australia, and USA, with various published data. The new enzyme immunoassay (ELISA) developed to test for Borna disease virus-specific circulating immune complexes, antigen, and free antibodies as the key marker triplet for determining infection in severe mood disorders has stated by Dr Liv Bode (2001).
The study will look at the increased prevalence and level of BDV markers in bipolar patients and correlate with the severity and cycling periods as a major indicator of the stage of the condition.

where appropriate, define the statistical power, e.g. 50% chance of detecting a 10% difference

So far, the ELISA triplet test is the most sensitive test used, with results about 10 times higher than other serological methods in use. The calculation is based on previous studies and this study is adequately powered.

c) whose advice have you taken?

The world leader experts in BDV, Dr Liv Bode and Prof. Hans Ludwig

❑12. Protocol - a full commercial protocol, a full research protocol submitted to a financing organisation (e.g. MRC) or a complete protocol for locally arranged research is required, as appropriate. If a questionnaire is to be used as part of the research project, a full copy must be included.
I've sent all I've got

13. What method is proposed for obtaining informed consent to participation? - enclose copies of Patient Information Document and Consent Form.(A routine hospital consent form is not normally appropriate for research projects).
❑ Consent form attached

❑

❑ 14.What is the regulatory status of the drug(s) to be used in the study?

No drugs are used
a) all drugs to be used within the terms of their existing product licence .....
b) study to be carried out under a DDX enclose copy .....
c) study to be carried out under a CTX or CTC enclose copy .....

Who will be responsible for the storage and dispensing of the Trial Drugs? Have the arrangements been discussed and agreed with that person?

No trial drugs involved
❑ 16. Which organisation will provide the indemnity for subjects in this study -

a) Has the sponsor confirmed, in writing, acceptance of the ABPI or ABHI 'Clinical Trial Compensation Guidelines'? - enclose copy YES/NO
b) The Trust - which Trust Chief Executive has agreed: We have written to the 3N's Chief Executive (enclosed)
c) University of Newcastle - have arrangements been agreed
with the Research Services Unit?:We have written to Digby Harris (enclosed) YES/NO
d) University of Northumbria - have arrangements been agreed
with the Legal Administrator?:Are you involved with UNN? yes
e) Other - please give details:

17. NHS-based project:
Is the Chief Executive aware of the proposed study and in agreement with it taking place on Trust premises with particular reference to the use of NHS resources and the financial arrangements?
We have asked his permission (letter enclosed)

18. University-based project:
Has the proposed study received approval via the appropriate University (e.g. 'Blue Form' for Newcastle) mechanism?
Yes

19. Payments:
a) will any payments be made to healthy volunteers?

Volunteers will be compensated for time and inconvenience
b) will patients/healthy volunteers out-of-pocket expenses be met?

Yes

c) details of financial support including commercial sponsorship to individual, department, hospital and/or university
Maria, we could apply for a few quid for this. Stuart, a bit of money is always most welcome

20. Planned start and finish dates for the proposed study:

a) Start As soon as ethical approval is given

b) Finish 2003

ALL APPLICATIONS

Summary in lay terms of the proposed research including details of drugs/treatment intended.
(Maximum length - 150 words)

Borna disease virus (BDV) is a neurotropic negative single-stranded RNA virus, recently classified and placed in the family of Bornaviridae within the order of mononegavirales. It infects a wide range of animal species ranging from birds to primates. In humans BDV may be implicated in the pathogenesis of some psychiatric disorders such as schizophrenia and bipolar disorders. Current methods for the detection includes, Nested reverse transcription-polymerase chain reaction (RT-PCR) for BDV RNA, ELISA for antibodies, antigens and immunecomplexes, and Indirect Immunofluorescence for antibodies raised against the virus. Epidemiological studies show that BDV nucleic acids are present with higher percentage (about 50%) in patients with schizophrenia and (about 40%) patients with bipolar disorder compared to patients with other disorders such as Alzheimer's disease, multiple sclerosis, or Parkinson's disease. These findings not only indicate that BDV may be a clear factor associated with schizophrenia and bipolar disorders, but also provides evidence of infection by the agent.
Previous work suggests that active infection is associated with relapse. We therefore intend to study bipolar patients in remission and relapse to further examine this association which has implications for our understanding of the pathophysiology of bipolar disorder.

Signature of Applicant: ..... date .....

Address for communications

Signature of Consultant/GP/Health Care Professional: ..... date .....

Print Name: .....

[Any research which is to be carried out within five or more LREC's geographical boundaries must be initially considered by the Multi-Centre Research Ethics Committee for the Region in which the principal researcher is based before subsequent referral to LRECs. Your signature on this form indicates that the research for which you are seeking ethical approval will be undertaken in no more than 4 LRECs' geographical boundaries.]

Please indicate the number of UK centres involved in this research: .....

Completed forms should be sent to:
Administrator, Joint Ethics Committee, Centre for Health Services Research
21 Claremont Place, Newcastle, NE2 4AA

tel: (0191) 222.5274

V3.1 February 2000